STUDIES OF LIPOPHILIC DEXTRAN GELS FOR THE CHROMATOGRAPHIC ANALYSIS OF STEROIDS

A thesis submitted in part fulfilment
of the requirements for admittance
to the degree of

Doctor of Philosophy

by

Robert Arthur Anderson

University of Glasgow

September 1973.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

For my parents.

ACKNOWLEDGEMENTS

The work described in this thesis was carried out from 1970 to 1973, with the joint support of the Departments of Chemistry and Botany, on a grant from the Agricultural Research Council. My sincere thanks are extended to Dr. C.J.W. Brooks and Dr. B.A. Knights for their constant advice and encouragement throughout the course of this study and for their willingness at any time to discuss various aspects of the work. I would also like to thank Professor R.A. Raphael, F.R.S., Professor G.W. Kirby and Professor M.B. Wilkins for the opportunity to carry out this research. I gratefully acknowledge the assistance given by the technical staff of the Chemistry Department and in particular by members of the GC-MS group.

Department of Chemistry,
University of Glasgow,
September 1973.

Part of the work described in this thesis has been published:-

(1) R.A. Anderson, C.J.W. Brooks and B.A. Knights, Preparation and evaluation of a chiral derivative of Sephadex LH-20. J. Chromatogr., 1973, 75, 247.

(2) R.A. Anderson, B.A. Knights and C.J.W. Brooks, Preparation and evaluation of a hydroxycyclohexyl derivative of Sephadex LH-20. J. Chromatogr., 1973, 82, 337.

(3) R.A. Anderson, C.J.W. Brooks and B.A. Knights, Hydroxyalicyclic derivatives of Sephadex LH-20 for lipophilic gel chromatography. J. Chromatogr., 1973, in press.

Reprints, where available, are enclosed in an envelope on the rear inside cover of the thesis.

C O N T E N T S

FOREWORD

References to literature publications are indicated by a simple superscript thus: "... a recent review,[5] ...". Figures are numbered according to the Chapter in which they appear. Thus Fig. 2.5 refers to the fifth figure in Chapter 2. The second section has not been subdivided into chapters. The Roman numeral II is used for paragraph headings and figures thus: para. II.1 etc.; Fig. II.1 etc. The appendices are numbered A.1, A.2 etc. The following abbreviations are used in the text:-

α	relative retention
$[\alpha]_D^{20}$	optical rotation at 20°C (sodium D-line)
F.I.D.	flame ionisation detector
GC	gas chromatography
GLC	gas-liquid chromatography
GLC-MS	gas-liquid chromatography - mass spectrometry
GPC	gel permeation chromatography
h	hour
HPLC	high performance liquid chromatography
$I_{OV\text{-}1}^{225}$	retention index on OV-1 phase at 225°C
i.d.	inner diameter (tubing)
IR	infrared
k'	capacity factor
K	equilibrium distribution coefficient
LC	liquid chromatography
LH-20	hydroxypropyl Sephadex G-25
LLC	liquid-liquid chromatography
m	metre
min	minute
N	number of theoretical plates
NMR	nuclear magnetic resonance
ϕ	peak capacity

ppm	parts per million
P.T.F.E.	poly-tetrafluoroethylene
R_s	resolution
R.I.	refractive index
RT	room temperature ($20^{\circ}C$)
σ	standard deviation of a Gaussian function
SEV	standard elution volume
SRV	solvent regain value
t_o	nonsorbed time (retention time of a nonretained compound)
t_{r1}	retention time of component 1
TFA	trifluoroacetate
TMS	trimethylsilyl
TMSE	trimethoxysilylethyl
UV	ultraviolet
V_{fs}	volume fraction of the stationary phase available to the solvent molecules
V_m	column void volume
V_r	retention volume
V_s	volume of the stationary phase
W_1	peak width of component 1.

SUMMARY

The work described in this thesis is concerned with the preparation of gel stationary phases for liquid chromatography in organic solvents. Sephadex dextran is a crosslinked polymer of D-glucose molecules. It contains many free hydroxyl groups which make it hydrophilic. Substitution of these groups with a suitable organic residue gives a lipophilic gel which swells in organic solvents and which is well suited for use as a chromatographic stationary phase. Gels were prepared with optically active substituents with the intention that selective phases would be produced which might permit the separation of enantiomers and phytosterols containing epimeric alkyl substituents on the side chain. No satisfactory chromatographic method of analysing such sterols has yet been reported.

A suitable method of attaching substituents (first reported by Sjövall and co-workers) was the BF_3-catalysed reaction of an olefin oxide with LH-20, a hydroxypropyl derivative of Sephadex G-25. A chiral oxide, 23,24-oxido-5β-cholane was prepared from naturally occurring bile acids. The conditions of the substitution reaction were optimised such that stoichiometric amounts of oxide were incorporated efficiently into the gel. A product containing 40% by weight of hydroxycholanyl residues (hydroxy-5β-cholanyl LH-20) was obtained. The physical properties of the gel were examined. Solvent regain values, sample recovery from the column, and the effect of sample size on the chromatographic elution profile were determined. The gel was then examined with respect to chromatographic properties in two solvent systems (benzene and methanol-heptane 9:1). These gave rise to straight-phase

or reversed-phase gel systems respectively. A model set of compounds, mainly steroids, was used in this evaluation.

The gel proved satisfactory as a stationary phase. Quantitative recovery of samples, symmetrical peaks, and an absence of tailing were characteristic of gel chromatograms. The straight-phase system was useful for the analysis of hydroxylic steroids. Alcohols, amines and carboxylic acids were all retarded on the column. Hydrocarbons, esters and ketones all had similar elution volumes and were not markedly retarded on the column. In the reversed-phase system , the order of elution was inverted, and non-polar compounds were retarded on the column relative to polar materials. This system gave rise to separations of substances differing in the hydrocarbon substitution. However, no separations were observed, in either system, of enantiomers or of phytosterols containing diastereomeric side chains.

A second derivative was prepared using cyclohexene oxide. This was a model compound for the reaction of more complex alicyclic oxides. The BF_3-catalysed substitution reaction was successful and marked the first occasion when an non-terminal olefin oxide had been incorporated into a gel. While generally similar to hydroxy-5β-cholanyl LH-20, this gel proved useful for the separation of epimeric steroids containing hydroxyl substituents and also 5α/Δ^5-steroids. Four other alicyclic oxides were reacted with LH-20. Two of these, 2α,3α-oxido-5α-cholestane and endo-2,3-oxidobornane were prepared from optically active starting materials. The gels were evaluated as chromatographic stationary phases. Improved separations of hydroxylic steroid epimers were obtained on hydroxycholestanyl LH-20. The properties of the four gels were generally similar to hydroxycyclohexyl LH-20. Neither enantiomers

nor phytosterol diastereoisomers could be separated on these phases. Some inferences are drawn on the mechanisms of separation on the gels, and suggestions are made for further work.

The second section deals with the analysis of the metabolites of cholesterol (labelled with 4-^{14}C-cholesterol) in the plant pathogenic fungus, Phytophthora cactorum. The major products were esters of cholesterol and long chain fatty acids. The fatty acid pattern was determined and compared with that of the fungal triglycerides.

SECTION I

STUDIES OF LIPOPHILIC DEXTRAN GELS.

Chapter 1. INTRODUCTION.

1.1 Chromatography: A General Introduction.

Since its inception at the start of the century, chromatography has enjoyed an expansion in proportion to that of chemistry as a whole. The diversity of its applications has caused it to become the most widely used separation method. Not the least of the reasons for this situation is the availability of a wide range of chromatographic systems. These are illustrated in Fig. 1.1. Two major points of subdivision arise, concerning the nature of the mobile and of the stationary phases. We shall be concerned primarily with those systems in which the mobile phase is a liquid.

Liquid-solid adsorption chromatography was the first type of system to be devised. Two workers, M. Tswett, a Russian, and D.T. Day, an American, independently devised chromatographic systems in which separations were achieved by the adsorption of solutes on the surface of an active solid contained in a column.[1] Of the two, Tswett is the more prominent figure. He recognised and correctly interpreted the separation process and also developed a practical laboratory procedure for its use. Although this type of chromatographic system is very widely used, it will not specifically be dealt with again except for incidental references.

The introduction of paper partition chromatography by A.J.P. Martin and co-workers[2,3] was the next major development in chromatography. In this system, solutes were separated by partitioning between the liquid mobile phase and the water trapped in the cellulose fibres of the paper. Organic liquid stationary phases can

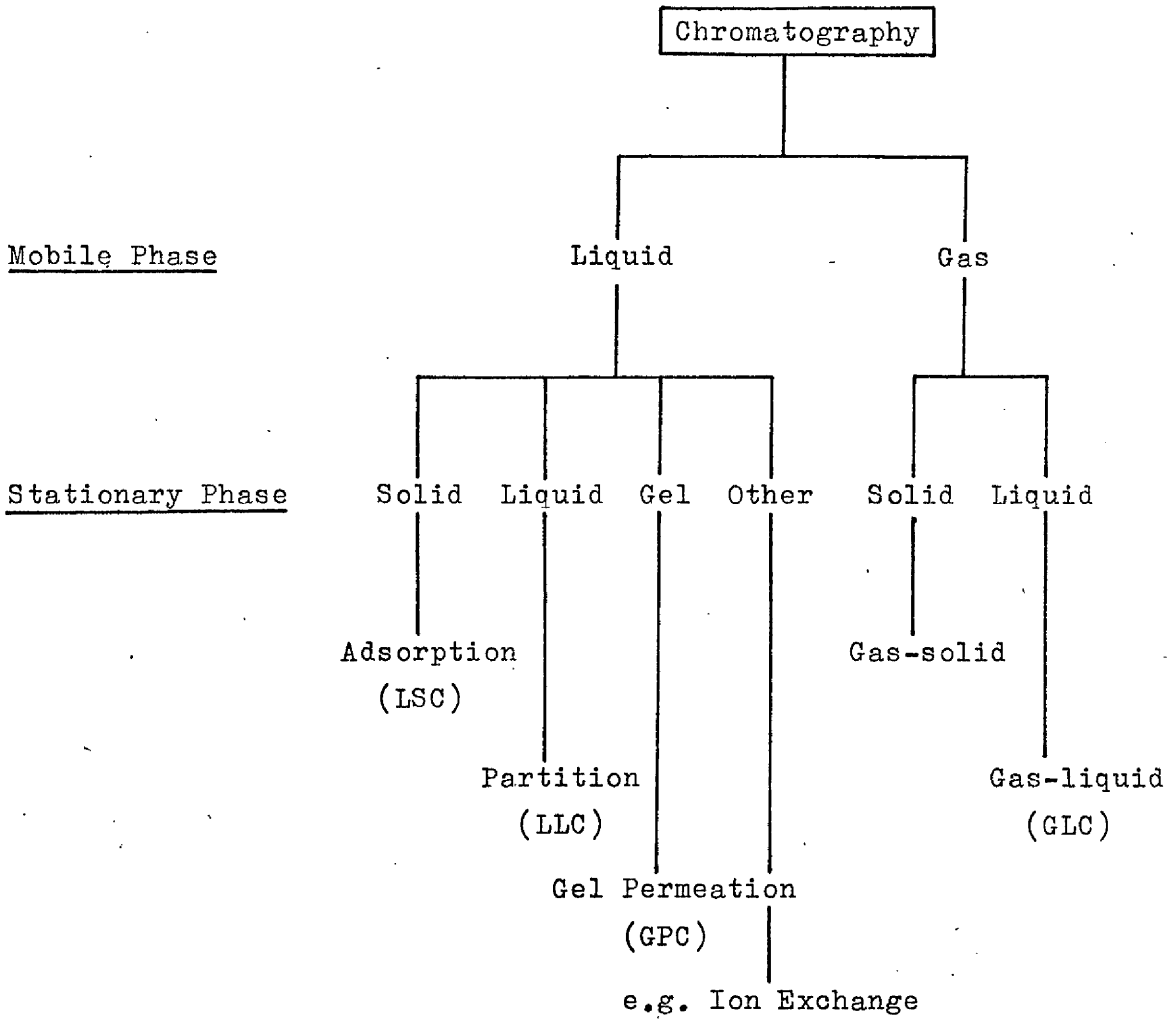
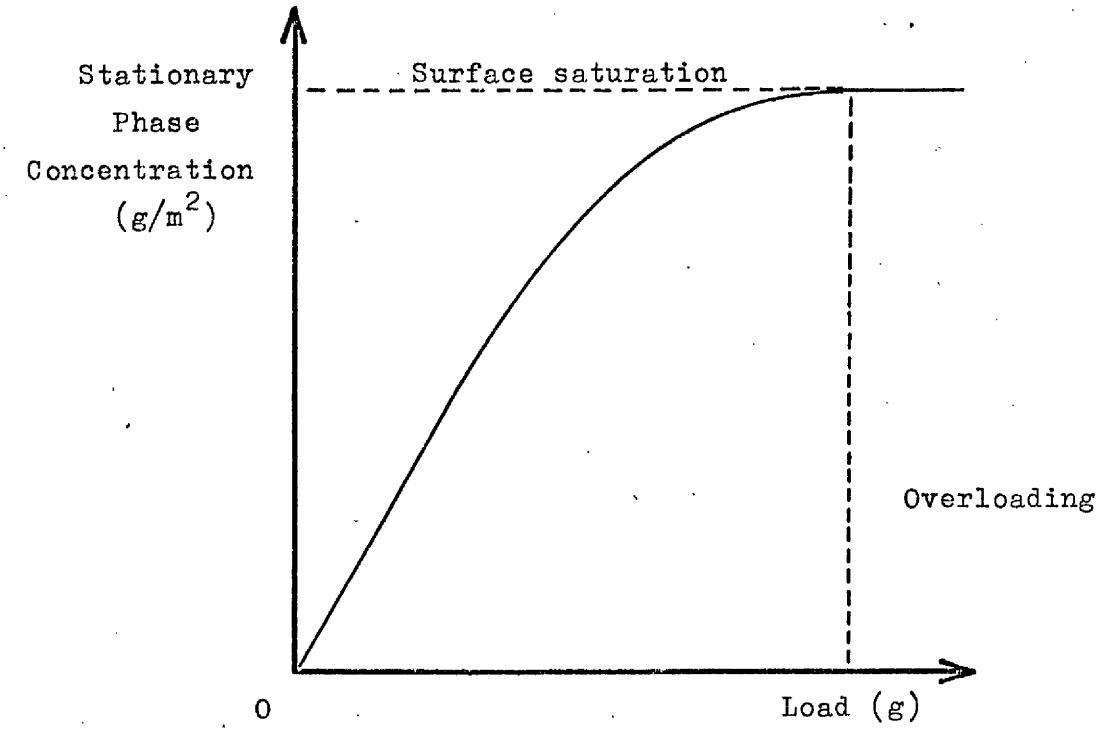
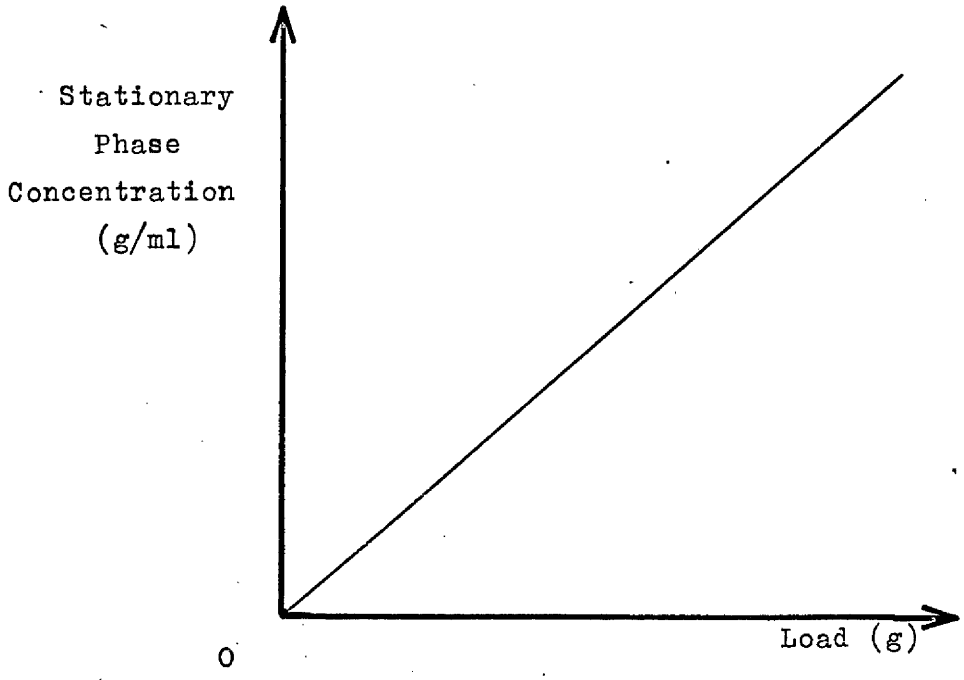


Fig.1.1 The subdivision of chromatographic systems according to the nature of the mobile and stationary phases.



(a)



(b)

Fig.1.2 Distribution isotherms for adsorption (a) and partition (b) systems.

be used when supported by a suitable inert solid. The concept of using a gaseous mobile phase in a partition system was also introduced by Martin,[2] but implementation of the idea followed much later.[4] The rapid development of gas chromatography which followed tended to eclipse the role of liquid chromatography as an analytical method and it is only in the last ten years that the latter has become prominent once more (para. 1.2 below). Partition systems can be subdivided according to the relative polarities of the mobile and stationary phases. Straight-phase systems are those in which the mobile phase is the less polar of the two, and reversed-phase systems are those having a stationary phase which is less polar than the mobile phase.

Gel permeation chromatography, alternatively called molecular sieving or gel filtration, is a type of chromatography in which molecules are separated according to their size. It will be dealt with in detail below (para. 1.5).

One of the characteristic features of a chromatographic system is the shape of the distribution isotherm. Thus an adsorption isotherm is often convex, (Fig. 1.2a) and overloading of the surface is sometimes seen even at low loads. A partition isotherm is generally a straight line (Fig. 1.2b). This is true if the partition coefficient is independent of the concentration. It is possible for more than one mode of separation to occur simultaneously in a chromatographic system. For example, adsorption and partition co-occur on alumina which has been deactivated with water, and partition and molecular sieving are together responsible for separations in some gel systems.

1.2 Modern Liquid Chromatography.

Recent developments in the field of liquid chromatography (LC)[5] have made this method attractive for the attempted solution of a wide range of analytical problems. Advances in technology and improvements in the stationary phases available have followed an understanding of the processes underlying chromatographic separations. Such insight had already been obtained in gas chromatography (GC), which, until recently, was almost unchallenged as the premier analytical method in organic chemistry. Outstanding problems associated with classical LC are the time required for an analysis and the low column efficiency. Long analysis times result in sample dilution by diffusion in the column and therefore samples must necessarily be larger than those required for GC to permit adequate detection.

The application to liquid chromatography of theory developed for gas chromatography must take into account four basic differences between liquids and gases:

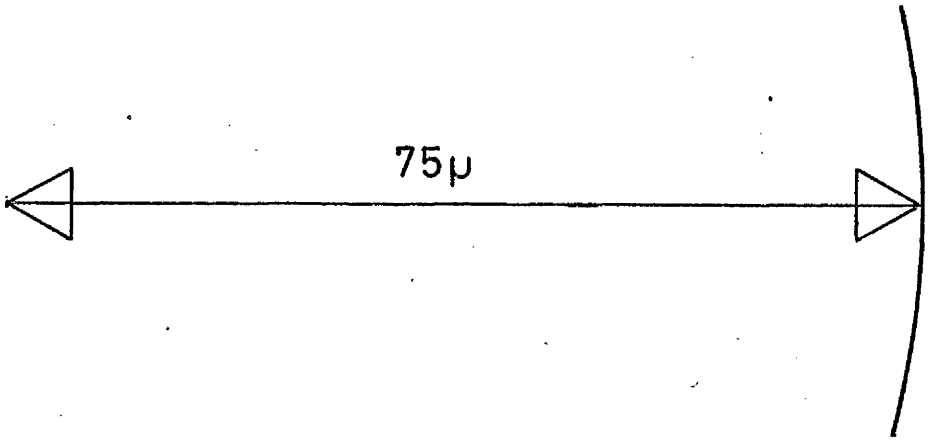
(a) liquids are approximately 100 times more viscous than gases;

(b) diffusion coefficients of solutes in liquids are at least 10^4 times smaller than the interdiffusion coefficients of gases;

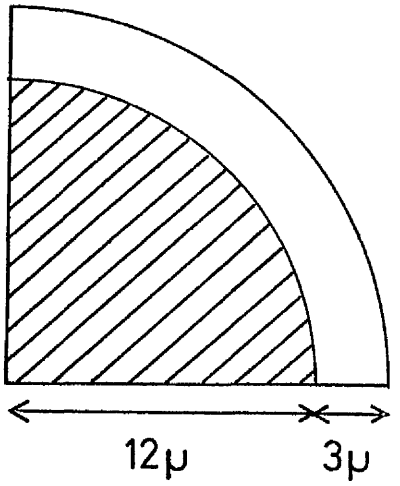
(c) the mobile phase will play a more prominent role in LC: interactions of the mobile phase with the sample and stationary phase are crucial to achieving separations, whereas in GC these interactions are not thought to be significant;

(d) liquids are incompressible under the conditions used in practice.

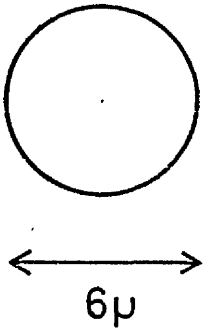
An appreciation of these differences gives an indication of how liquid chromatographic analysis might be improved. Thus, because



Classical liquid chromatography particle



Controlled surface porosity bead



Silica microsphere

Fig.1.3 Relative dimensions of liquid chromatography particles.

viscosities are 100 times higher in liquids, the applied pressure must be at least 100 times higher than that used in GC to obtain comparable mobile phase velocities (i.e. approximately 1 cm/sec as opposed to 0.001 - 0.01 cm/sec in classical LC). Small diffusion coefficients in liquids result in a high resistance to mass transfer between mobile and stationary phases and therefore lead to low column efficiency at high mobile phase velocities. Classical liquid column packings used porous particles with large surface areas, useful for adsorption chromatography, but the deep pores contained within resulted in a large pool of stagnant mobile phase and in long solute migration distances. Under such circumstances, mass transfer effects limit the speed of analysis that can be achieved, if reasonable efficiency is to be maintained. Clearly, the pore depth must be reduced. Two approaches have been made (Fig. 1.3):

(a) The use of controlled surface porosity beads. The maximum pore depth is limited by the thickness of the porous layer surrounding the solid core. Originally proposed by Golay[6] and Purnell,[7] these materials were proved successful by Halász,[8] and commercial preparations are now available, for example Corasil I and II.[9]

(b) Silica microspheres.[10] Formed by coagulation of microparticulate silica, these spheres have very small diameters (6μ). Mass transfer is therefore rapid throughout the solid.

The particle sizes used in liquid chromatography have tended to become smaller (<40μ, compared with classical packed columns using 150μ particles). This has resulted in an increased resistance to solvent flow. Operating pressures of liquid chromatographs are currently in the range 500 - 3000 psi or higher (up to 5000 psi has been used).

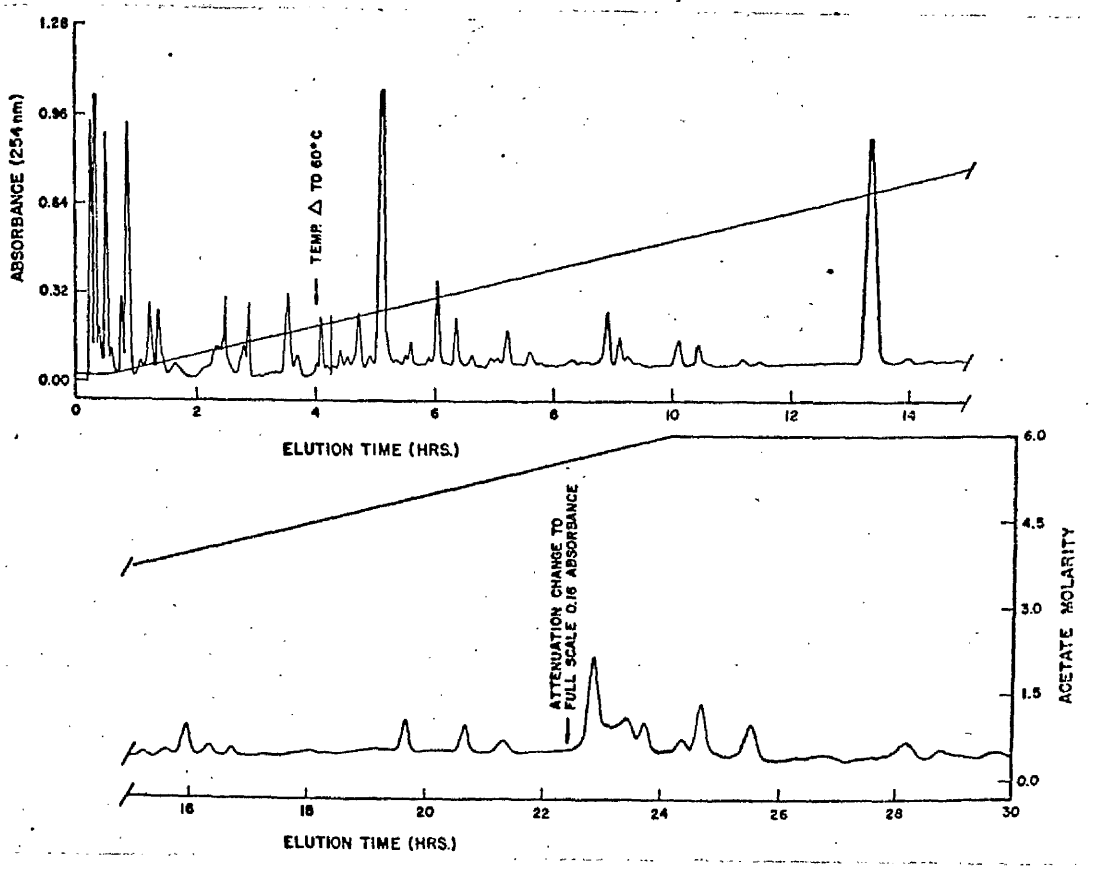


Fig.1.4 UV-absorbing constituents of human urine. Column = 100 cm, 2.4 mm i.d., strong anion-exchange resin, gradient elution. (Reproduced from Ref.15).

Undoubtedly the advances in stationary phase design have been responsible for the re-awakening of interest in liquid chromatography. Liquid-liquid chromatography has advanced following the development of surface-reacted (bonded) stationary phases and of gel phases. In the former, siliceous supports are esterified with a monomolecular organic layer of alcohols ("brushes")[11] or the surface of the support is reacted with silane reagents which are then polymerised to give a silicone coating.[12] Gels also involve a liquid-liquid partition and these will be dealt with below (para. 1.5) in more detail.

As a result of these innovations, column efficiencies in LC have been markedly improved. The resolving power of columns packed with porous layer beads or silica microspheres is almost equivalent to that of classical packed GC columns, and Karger predicts[5a] that high speed liquid chromatography will soon compare favourably with GC in terms of speed of analysis. The scope for applying LC is potentially larger than for GC. Samples for the latter are limited to those volatile enough (and having sufficient thermal stability) to permit their partition into the mobile phase without decomposition and without having an excessive retention time. Liquid chromatography can cope with samples of any description - provided they can be dissolved in a suitable liquid - and separations are normally achieved at room temperature.

The diverse fields in which LC has been applied include pharmaceuticals[5b] (analgesics, antibiotics), agriculture (herbicides, pesticides), quality control (dyestuffs,[5b] polymers[13]) and clinical chemistry.[14] One example in the last field is shown in Fig. 1.4, which is a liquid chromatogram of an untreated urine sample, run on a strong anion exchange resin.[1c,15] 90 peaks were observed in the course of the

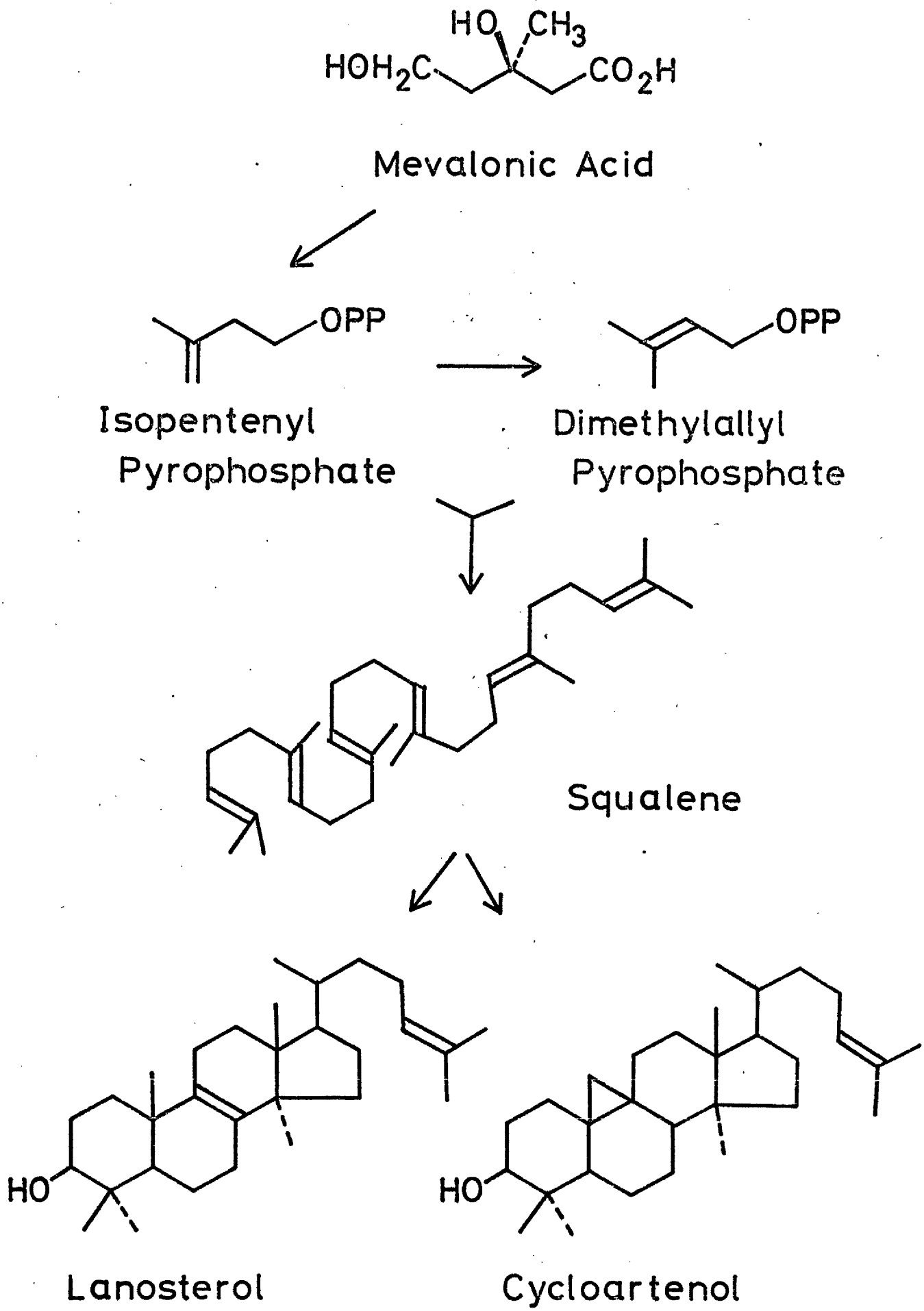
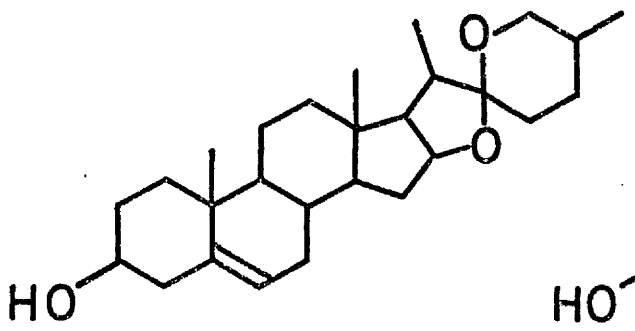
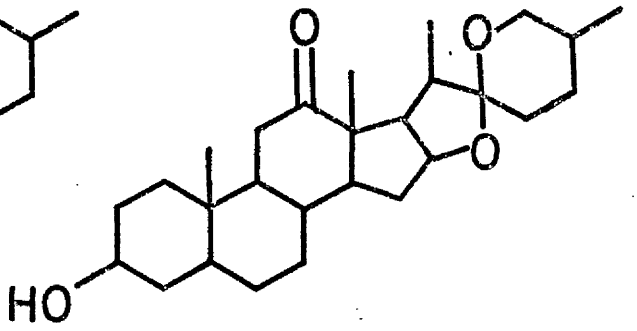


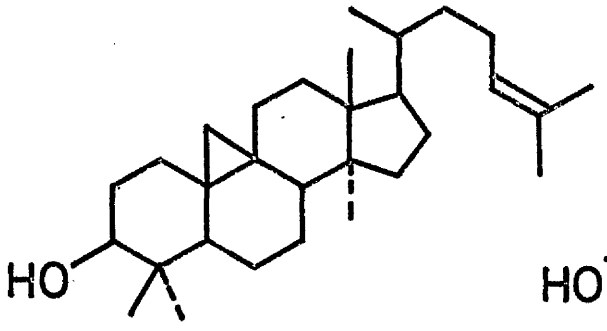
Fig.1.5 Biosynthesis of plant and animal steroids.



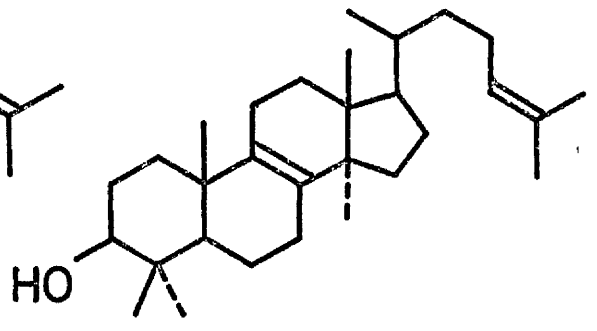
I Diosgenin



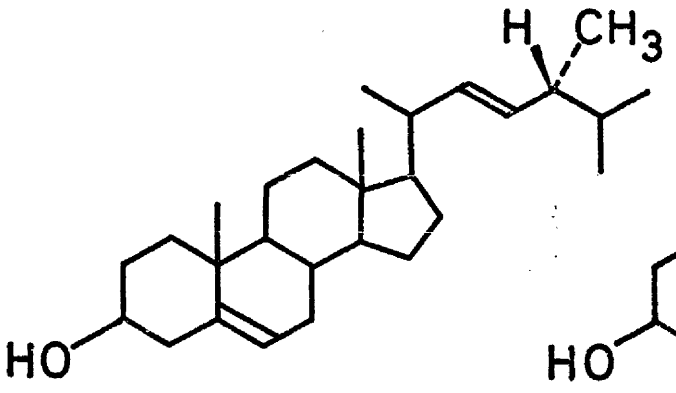
II Hecogenin



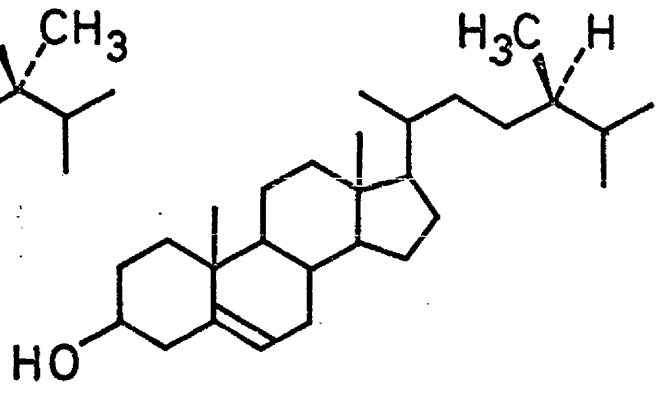
III Cycloartenol



IV Lanosterol



V Brassicasterol



VI Campesterol

30-hour run. Further examples include analyses of steroid hormones (see for example reviews by Bush[16] and Morris[17]). Also, high speed LC has successfully been applied to the separation of steroids by several workers.[18-20] The following paragraph deals with a difficult problem in phytosterol analysis which it was hoped might be solved by liquid chromatography.

1.3 The Problem: Phytosterol Analysis.

Steroids in animals have been known for some time to have at least three functions - as precursors of other steroids, as membrane components, and as hormones.[21] The last function has stimulated much work in the pharmaceutical industry on the synthesis of steroidal analogues for a variety of medicinal purposes. An increased demand for resources of steroidal material is virtually ensured by the success of oral contraception alone, and the most abundant source of steroids is the plant kingdom. Steroidal sapogenins, for example diosgenin (I) and to a lesser extent, hecogenin (II), are readily available and these can be converted to suitable materials for industrial syntheses of hormone analogues. The role of steroids in plants has been compared to that in animals:[22] they are known to form part of the biological membranes in plant as well as animal cells,[23,24] and the overall mode of biosynthesis seems to be common to both plants and animals (Fig. 1.5). Evidence is accumulating for a hormonal function of steroids in plants, a role which was not previously appreciated. For example, estrogens have been detected during flower bud formation and development in bean (Phaseolus vulgaris) plants,[25] and also, estradiol and testosterone were observed to induce femaleness in monoecious cucumber plants.[26] Sterols are required (from the growth medium or host plant) for sexual reproduction

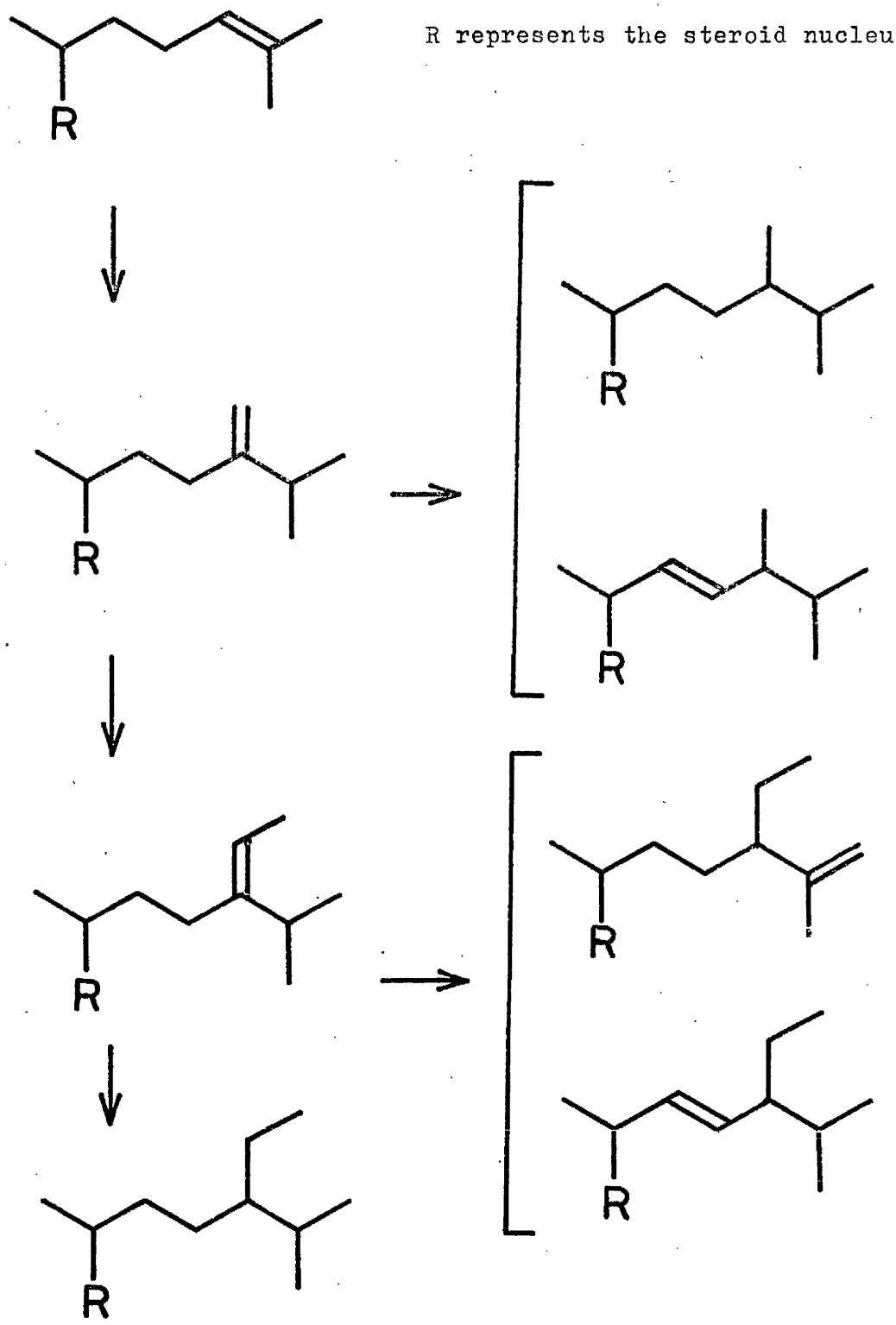


Fig.1.6 Side-chain modification processes for phytosterols. Some alternative steps exist which are not shown in this diagram.

to occur in Phytophthora and Pythium species, as these fungi are unable to synthesise steroids. Sterols in roots of the snapdragon (Antirrhinum majus) appear to confer disease resistance on the plant by decreasing the activity of the pectic enzymes responsible for the pathogenic action of Pythiaceous fungi.[29]

Phytosterols are formed from mevalonic acid (MVA) by a reaction pathway similar to that in animals (Fig. 1.5). Some differences exist in the metabolism of steroids in plants compared to animals, for example, the immediate product of cyclisation from squalene is cycloartenol (III) and not lanosterol (IV). Also, side-chain modifications occur giving phytosterols their familiar C-24 substituents[30] (Fig. 1.6). The alkylation step is stereoselective: either the R- or the S- isomer is formed and, as far as is known, not a mixture of the two configurations. The configuration which is found appears to depend on the species of plant examined, and this may be of taxonomic significance.[31] Thus in higher plants, 24α- substituents are found, while lower plants - green algae and fungi - have 24β- substituted sterols,[32] although a few exceptions exist, for example, the group of Δ^{25}-sterols and also brassicasterol (V), all of which have the 24β-configuration. In the first example, this is probably due to the mode of biosynthesis.[33]

While analytical methods are available for the separation, purification and identification of substances from complex biological extracts, it has often proved difficult or impossible to establish the stereochemistry at the 24-position of phytosterols, or to relate it to the known configuration of standard materials. The techniques of IR spectroscopy, mass spectrometry (MS), and GC have so far been unable to distinguish diastereoisomers of this type. Optical rotation methods have been applied

but the errors involved tend to detract from the credibility of conclusions reached. Thompson showed[34] that high resolution NMR spectroscopy could differentiate between 24α- and 24β- isomers but this method is not generally available and is unsuitable on a small scale. Chromatographic resolution of these closely similar compounds would be a more favourable solution to the problem.

Many cases in which this problem has arisen are studies of phytosterols in plants chosen because they are suitable from an experimental viewpoint, even though the stereochemistry of the sterols at the 24-position is unknown. One such study involves brassicasterol.[35] This compound has been found in species of the Cruciferae in admixture with campesterol[31] (VI). Present evidence indicates that these have opposite configurations at C-24.[36] The assumption was made that the C_{28} $\Delta^{5,22}$-sterol of the Cruciferae was brassicasterol (originally isolated from Brassica rapa[37,38]) and that the C_{28} Δ^{5}- sterol was campesterol (originally obtained from Brassica campestris[39]). The most likely interpretation of this evidence is that separate enzyme systems are responsible for the biosynthesis of the two sterols.[31] The apparent involvement of brassicasterol in seed development of species of the family Cruciferae[35] indicates that this sterol has an important function in these plants, and this lends support to the postulation of separate enzyme systems. A recent chemotaxonomic survey of a number of species of the Cruciferae distinguished various groups according to the types of phytosterol present.[31] The stereochemistry at C-24 (when both C_{28} and C_{29} Δ^{22}-sterols were present) could not be determined. If the C_{28} and C_{29} sterols had the same configuration (24β) then this would be the first recorded discovery of a Δ^{22}-(24β)-C_{29} steroid in a higher plant. If they were of

opposite configuration, further support would be lent to the idea that brassicasterol has a distinct biosynthetic pathway.

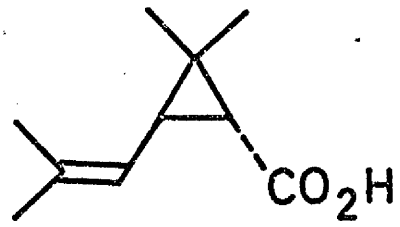
The ability to solve problems of this nature will help in our understanding of the role of sterols in plant biochemistry, and if this is as far reaching as that of steroids in animals, then it will be of considerable importance. The present work was undertaken with the aim of developing chromatographic methods of analysing these sterols.

1.4 Chromatographic Resolution of Chiral Molecules.

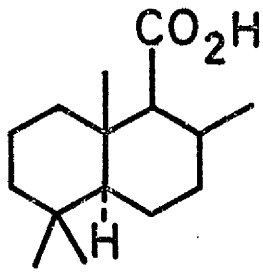
The problem of resolving enantiomeric and diastereomeric mixtures is recurrent in various fields of biological and medicinal chemistry and is becoming increasingly important in industry, for example where synthetic pharmaceutical products must be purified before use. The classical methods of resolving enantiomers by formation of diastereomeric salts have recently been reviewed.[40,41] Diastereoisomers tend to have slightly different physical properties, for example, melting point or solubility in a given solvent system. The latter is the basis for fractional crystallisation methods of resolution. However, such differences in physical properties are also the basic requirements for the chromatographic separation of two compounds. A gas chromatography column has a much higher number of theoretical plates (see para. 1.5 for an explanation of this term) than a distillation column for the separation of compounds of slightly different boiling point. Chromatography has been used, therefore, to provide a solution to many current resolution problems.[42] Invariably, one of two approaches has been used:-

(i) Chromatography of diastereoisomers.

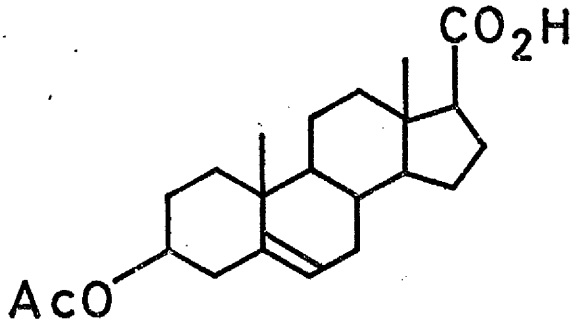
Diastereomeric compounds are formed from enantiomers by chemical



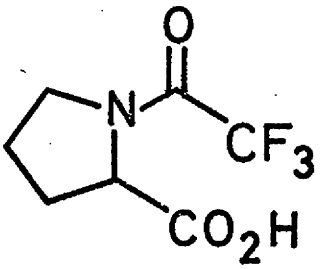
VII Chrysanthemic acid



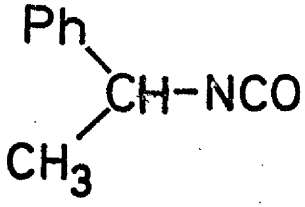
VIII Drimanoic acid



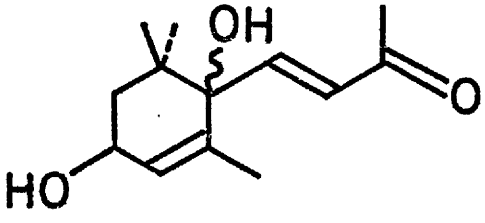
IX 3β-Acetoxy-5-etienic acid



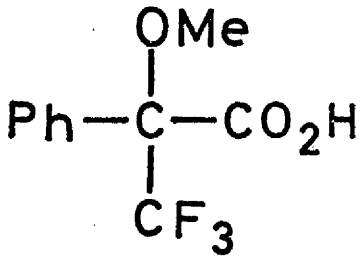
X N-TFA-Proline



XI Phenylethyl isocyanate



XII



XIII α-Methoxy-α-trifluoromethyl phenylacetic acid

reaction with a suitable optically active resolving agent and then chromatographed on normal stationary phases. Differences in the volatility or partition (or adsorption) coefficients of the two compounds permits GC or LC separations respectively. This approach has been used frequently in gas chromatography.[43-54] Resolving agents include chrysanthemic acid (VII) and drimanoic acid (VIII),[43] 3β-acetoxy-5-etienic acid (IX),[44] N-TFA-(S)-(-)-proline (X),[45] and (R)-(+)-phenylethylisocyanate (XI).[46] In LC, for example, bisnoronoceranediol menthoxyacetates[55] and spirononanediol (A) - camphanoates[56] have been separated. Also, Koreeda recently separated the diastereomers formed between XII and XIII by recycle chromatography on a porous silica ("Porasil") column.[57]

(ii) Chromatography of enantiomers.

Enantiomers have been resolved by chromatography on an optically active stationary phase.[40-42,58] The latter are often polymers prepared from optically active monomers, for example cellulose or dextran, or else the polymer matrix has been substituted with optically active residues. Silica gel has been modified by reaction of sodium silicate with (+)-camphorsulphonic and the resulting column used to separate (±) camphorsulphonic acid.[59] As an enantiomeric mixture passes through such a column, transient associations occur between the solute and the stationary phase. The diastereomeric complexes formed thus in situ will have different stabilities, and the mobilities of the two components of the racemate will be different. Three points of bonding or molecular association (for example of two aromatic rings, one in the solute and one in the sorbent) will be required to distinguish two enantiomers, a concept proposed some time ago to explain stereospecificity

in enzyme reactions.[60]

Many examples are recorded in the literature of the application of this approach in both gas and liquid chromatography. Gil-Av, Oró, and several other workers have established GC techniques for the analysis of amines and amino acids[61-65] using dipeptide-based stationary phases. In liquid chromatography, optically active polymers have been used, for example cellulose[58] and Sephadex dextran,[66] in aqueous media for the resolution of organic acids. Polymers modified by the addition of amino acid substituents, polystyrene[67] and Sephadex[68] have successfully been applied to the resolution of amino acids.

In all of the examples cited above, resolution of enantiomeric molecules on optically active phases has required three points of bonding between solute and sorbent. Resolution of diastereoisomers has been successful only when distinct differences occur between the two isomers with respect to a chromatographically important physical parameter. The problem of the phytosterol diastereomers does not apparently fit into either of these categories. The chiral grouping on the side chain contains either no functional substituents or only one (for example the Δ^{22} bond). Separation cannot be achieved, therefore, by use of a polar optically active stationary phase. Any differences existing in the physical properties of these steroids appear to be too small to effect chromatographic separation on conventional columns. The work described in this thesis was concerned with the preparation of stationary phases which would effect separations by means of a purely lipophilic interaction. Earlier investigations had shown that lipophilic dextran gels allowed the separation of lipids by this type of interaction especially when reversed-phase systems were used. The background to gel

TABLE 1.1 Materials for use in gel chromatography.

Soft		Semi-Rigid		Rigid	
Organic	Aqueous	Organic	Aqueous	Organic	Aqueous
2% Cross-linked polystyrene	Polydextran	Polystyrene	Polystyrene	Glass	Glass
Chemically modified polydextran	Polyacryl-amide	Polyvinyl acetate	Sulphonated Merrifield derivative	Silica	Silica
Rubber	Agarose	Polymethyl methacrylate		Modified silica	Modified silica
	Starch				

chromatography in general and to lipophilic dextran gels in particular will now be reviewed.

1.5 Gel Chromatography: A General Introduction.

Gel polymers are prepared by cross-linking polymer chains with a suitable reagent. The resulting giant molecule, while insoluble, will usually absorb (i.e. swell in) solvents in which the original polymer was soluble. As the number of cross links increases, the degree of swelling in an appropriate solvent falls. Three types of gel may be distinguished according to the amount of solvent imbibed by the gel (Table 1.1):-

(a) Soft gels: these swell to many times the dry volume of the gel. They are readily deformed by pressure and therefore are unsuitable for high-pressure LC. When operated at low flow velocities, soft gels offer high efficiency and capacity. Because of the large volume change in swelling, the pore size is highly dependent on the solvent. Traditionally, soft gels have been used in aqueous systems. Examples are polydextran ("Sephadex"), agarose ("Sepharose"), starch, and polyacrylamide.

(b) Semi-rigid gels: these swell to only 1.1 - 1.8 times their dry volume. The permeability and capacity are less than for soft gels, but semi-rigid gels can operate at high pressures without deformation. Currently available gels, for example the cross-linked polystyrene, Styragel, are used primarily in organic solvents.

(c) Rigid gels: these do not swell in solvent. Strictly speaking, some of the members of this group are glasses rather than gels, but the similarity of the separation processes occurring logically requires their

inclusion. Rigid gels can be used at high pressures and in either aqueous or organic solvent systems. An example is the porous silica, "Porasil".

Prior to a discussion of the mode of action of gels, some terms of general relevance to liquid chromatography will now be introduced. The classical chromatography equation describes the retention volume, V_r:-

$$V_r = V_m + K\,Vs \qquad \text{(Equation 1.1)}$$

where V_m = the column void volume

V_s = the volume of stationary phase (for liquid-liquid partition). In adsorption chromatography, Vs is replaced by a term relating to surface area or weight of adsorbent.

K = the equilibrium distribution coefficient (concentration of the sample in the stationary phase/concentration in the mobile phase).

An important retention parameter in chromatography is the capacity factor, k', defined as:

$$k' = K \cdot \frac{V_s}{V_m} = \frac{\text{amount of solute in the stationary phase}}{\text{amount of solute in the mobile phase}} \qquad (1.2)$$

When $k' = 0$, it is seen from equation 1.1 that $V_r = V_m$. As k' increases, the sample spends longer in the stationary phase compared to the mobile phase and therefore the retention time increases. Combining equations 1.1 and 1.2 gives:

$$V_r = V_m\ (1 + k') \qquad (1.3)$$

Since volume is proportional to time for constant flow rates, we have,

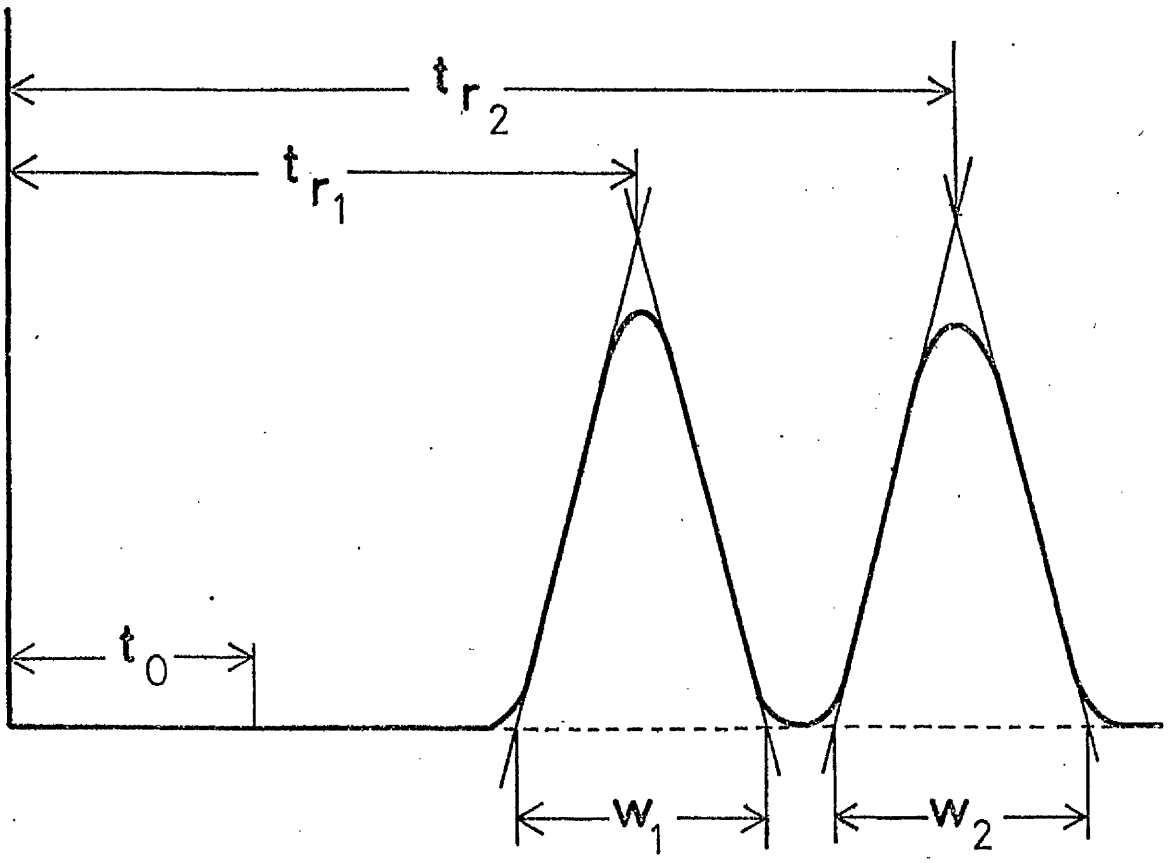


Fig.1.7 Chromatogram of a two-component mixture, illustrating retention time, t_r, nonsorbed time, t_o, and band width, W, of the Gaussian peaks.

on substituting for V and rearranging:

$$k' = \frac{t_r - t_o}{t_o} \qquad (1.4)$$

t_r and t_o are defined by Fig. 1.7, which illustrates a chromatogram of two compounds, indicated by the subscripts 1 and 2. The resolution of the two peaks, R_s, can be written as:-

$$R_s = \frac{2\,(t_{r2} - t_{r1})}{W_1 + W_2} \qquad (1.5)$$

A more useful equation relates R_s to the fundamental chromatography parameters (1.6). It is derived on the assumption that $W_1 = W_2$, a reasonable approximation for closely spaced peaks[69]

$$R_s = \tfrac{1}{4}\,\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k_2'}{1+k_2'}\right)(N_2)^{\frac{1}{2}} \qquad (1.6)$$

where α = the relative retention

N = the number of theoretical plates (the subscript 2 refers to the second component).

The relative retention, α, is defined by:

$$\alpha = \frac{t_{r2} - t_o}{t_{r1} - t_o} = \frac{k_2'}{k_1'} = \frac{K_2}{K_1} \qquad (1.7)$$

That is, α is the ratio of the equilibrium distribution coefficients of the two components. It is a measure of the thermodynamic differences in their distributions:

$$\Delta\,(\Delta G^\circ) = -RT \ln\alpha \qquad (1.8)$$

where $\Delta\,(\Delta G^\circ)$ is the difference in free energies of distribution for the two components. When $\alpha = 1$, the resolution is zero, regardless of the

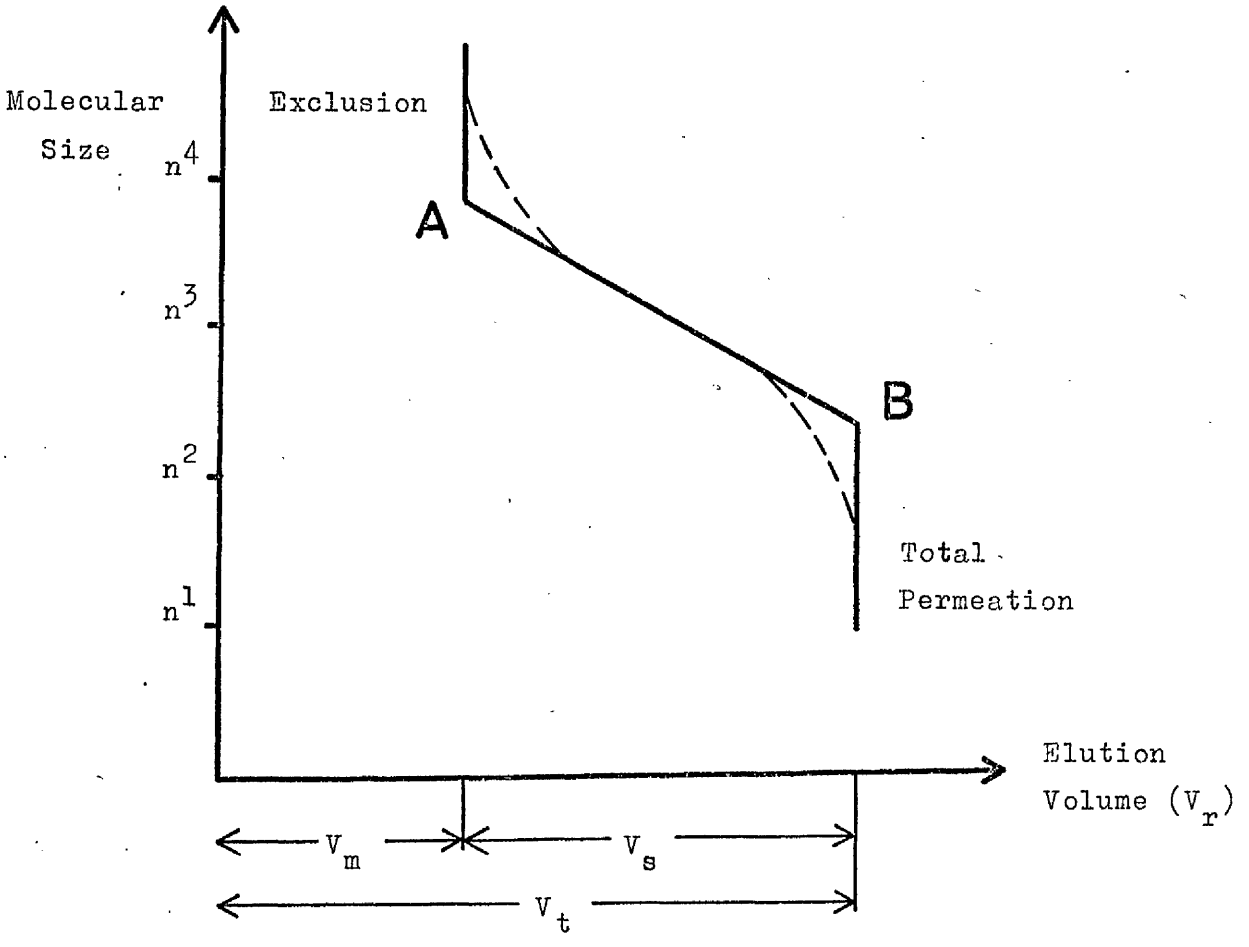


Fig.1.8 Graph of elution volume versus molecular weight for a gel permeation chromatography column. The solid line is the graph predicted by theory. The dotted curve represents that found in practice. V_m is the interstitial volume (volume of the mobile phase between the gel particles in the column), V_s is the volume occupied by the gel phase, and V_t is the total bed volume of the column.

value of N. For a separation to be possible, there must be a difference in the thermodynamic distribution behaviour of the two components; α, therefore, is a measure of the column selectivity towards the two compounds. In some forms of chromatography, separations can be achieved for α-values as low as 1.01.

The number of theoretical plates, N, is a measure of column efficiency. It relates to the band broadening in the column after a given time of migration:

$$N = 16 \left(\frac{t_r}{W} \right)^2 = \left(\frac{t_r}{\sigma_t} \right)^2 \qquad (1.9)$$

where σ equals the standard deviation of the Gaussian function in time units, assuming a Gaussian elution profile is obtained.

Two types of separation process are generally recognised in liquid-gel chromatography: gel permeation chromatography (GPC) and partition chromatography.

(a) Gel Permeation Chromatography is a type of liquid chromatography in which, characteristically, molecules are separated on a size basis. Retardation on the gel column results from diffusion of the solutes into the pores of the gel. Depending on the molecular size of the solute, a greater or lesser proportion of the included volume of the gel will be accessible. Larger molecules will therefore be eluted before small molecules. Fig. 1.8 illustrates a graph of elution volume versus molecular weight. The lower limit (A) is set by the column void volume V_m, which is the elution volume of molecules which are excluded from the gel. The upper limit (B), is the point at which all of the imbibed solvent is accessible to the solute. In practice, the graph is not straight but is curve (broken line Fig. 1.8). This arises because the pores in the gel

have a range of sizes, and because solutes of the same molecular weight can have different molecular volumes according to the conformations adopted.

For GPC, the retention equation (1.1) is rewritten as:

$$V_r = V_m + K_o V_s \qquad (1.10)$$

where $K_o = K(V_{fs}/V_s)$. V_{fs} is the volume fraction of the stationary phase available to the solvent molecules. When only gel permeation is taking place, the solvent in the pores is identical to that in the mobile phase and consequently K will be 1. K_o will have a maximum value of 1 (at which value, the upper limit B is reached, Fig. 1.8). When K_o (or K) exceeds unity, the elution volume is greater than $(V_m + V_s)$, and processes other than permeation are occurring. Two further differences arise between GPC and other forms of chromatography:

(i) Peak widths are equal at all values of K_o, a result of two compensating interactions.[70] Large molecules are eluted early but have low diffusion coefficients which causes peak broadening. Small molecules spend longer in the column. Both of these effects appear to lead to equal band broadening during a chromatogram.

(ii) The peak capacity (ϕ) of a GPC column is lower than for other forms of chromatography. This function is defined[71] by equation 1.11:

$$\phi = 1 + 0.6\, N^{\frac{1}{2}} \log (1 + k') \qquad (1.11)$$

It gives a measure of the number of peaks that can be placed within a certain time period in which all of the bands have a resolution of unity (i.e. 4σ), assuming a constant number of theoretical plates for each band. As K_o never exceeds unity, k' is necessarily low ($k' = K_o \frac{V_s}{V_m}$), having values of 3 or less compared, for example, with GC where values of 100 or more are known. ϕ is therefore lower in GPC than in

GC for columns of equal N.

(b) Partition Chromatography: In this form of liquid-liquid chromatography, the stationary phase within the gel pores is a liquid of different composition from the mobile phase. Separations are achieved as a result of differences in the partition coefficients (K) of the solutes in a mixture. Partition chromatography was first developed by Martin and Synge and co-workers,[2,3] who derived the relation

$$K = \frac{A_m}{A_s} \cdot \left(\frac{1}{R_f} - 1\right)$$

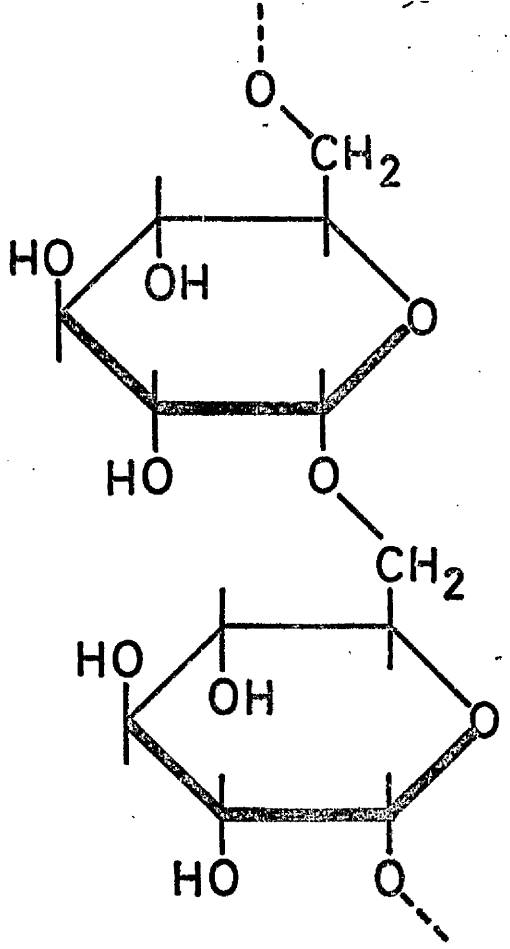
where R_f is the R_f value of the solute in the system and A_m/A_s is the ratio between the cross-sectional areas of the mobile and stationary phases. Detailed theoretical discussions of liquid-liquid partition are given by Locke,[72] Morris,[17] and Giddings.[73] As we have already noted for gel chromatography, A_m/A_s will vary according to the size of the solute molecules. The stationary phase in gel partition chromatography may be formed by preferential absorption of some solvents from a mixed-solvent mobile phase, giving rise to different compositions within and without the gel particles. Alternatively, if a single solvent system is employed, the stationary phase can be regarded as containing the gel in "incipient solution".[74] The imbibed solvent will be a mixture, effectively, of free solvent molecules and solvated polymer chains, and also, in some cases, solvated substituents on the polymer chains. A similar situation arises with chemically bonded supports for chromatography (Para. 1.2), in which case the solvated substituents on the surface of an inactive support provide a bound stationary

phase for liquid-liquid chromatography.

1.6 Lipophilic gel chromatography.

In this section, and subsequently, only soft gels will be under discussion. Semi-rigid gels such as the cross-linked polystyrenes are therefore excluded. (For recent reviews see refs. 5 and 75).

The majority of organic compounds are only slightly soluble in water, therefore to use liquid-gel chromatography for their separation non-polar gels and solvents are required. Straight-phase, reversed-phase and gel filtration systems can be obtained with lipophilic gels, depending on the relative polarities of the mobile and stationary phases. Early examples of reversed-phase chromatography are the separation of fatty acids on rubber-impregnated paper,[76] and of lipids on acylated paper.[77,78] Hirsch obtained good separations of non-polar lipids on columns of soybean factice[79] (polymerised and granulated soybean oil). In this case, a miscible solvent system was used and, after allowing the column to equilibrate, a stable system was obtained which allowed repeated use of the columns over a prolonged period. The stationary phase was probably formed by selective absorption of solvent into the matrix. A partially esterified cation exchanger was used by Seki[80] for the separation of steroids, and more recently oleophilic ion-exchange polymers have been described by Gregor and co-workers.[81] Vulcanised rubber latex has been used for gel permeation chromatography in organic solvents.[82]



Dextran Polymer

(Dex-OH)

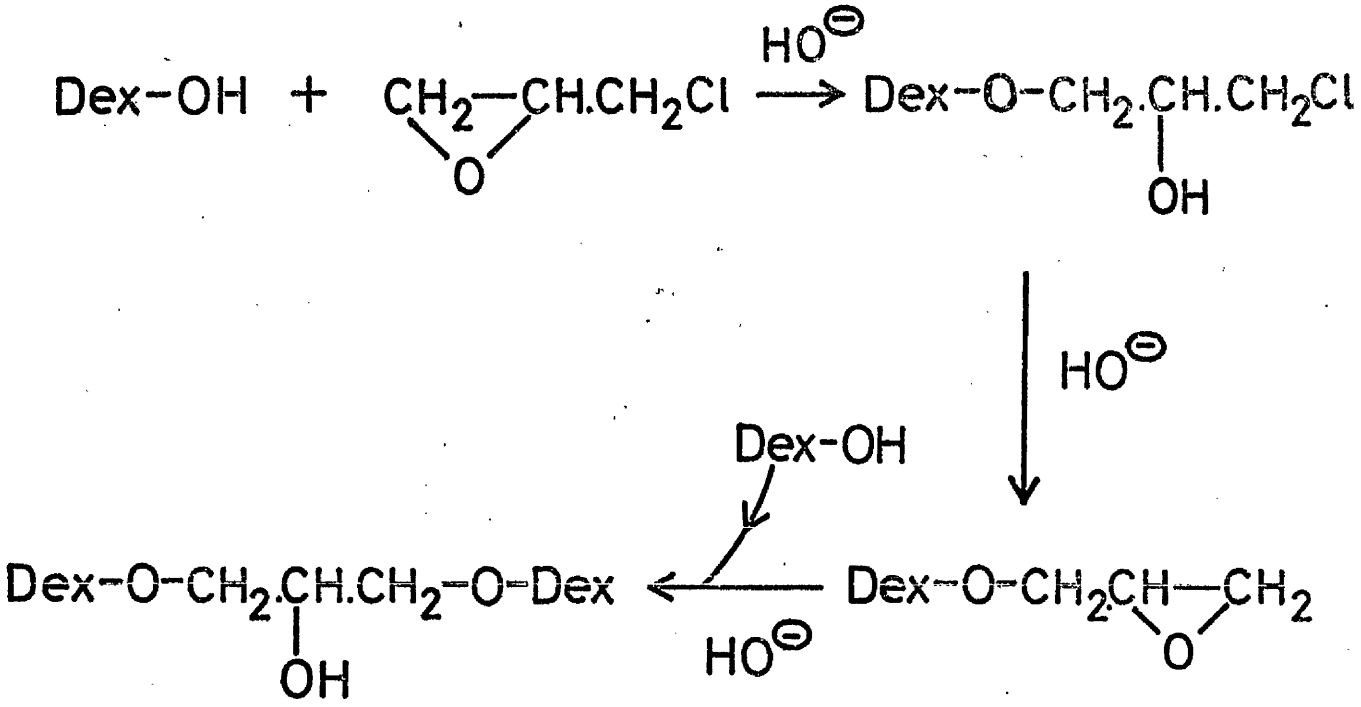


Fig.1.9 Dextran structure and the crosslinking reaction with epichlorhydrin (3-chloro-oxidopropane).

One of the more important recent developments in lipophilic gel chromatography has been the demonstration of the utility of dextran gels as stationary phases. In particular, advances made by Sjövall and co-workers[83] have provided the lipid chemist with a very versatile technique.

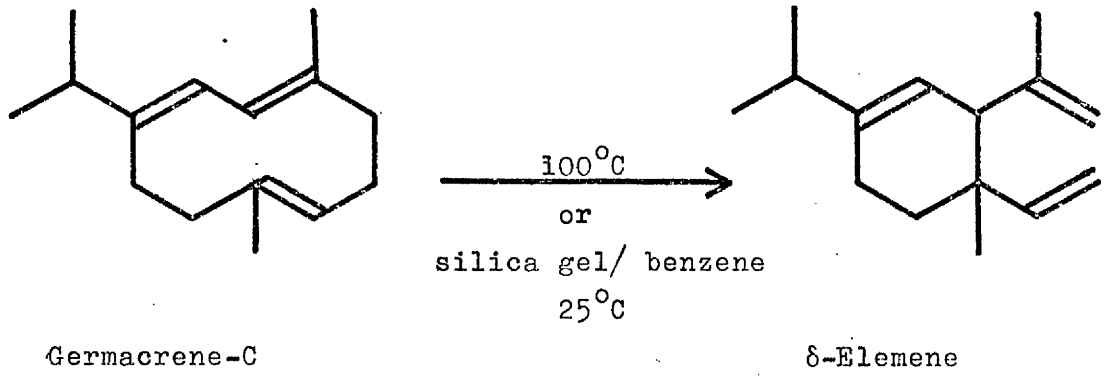
Sephadex is a commercially available polydextran formed by the cross-linking of dextran B512 with epichlorhydrin (Fig. 1.9). Dextran itself is composed of D-glucose molecules ether-linked through the hydroxyl functions at C-1 and C-6. Several varieties are marketed with different degrees of cross-linking[84] (G10, G15, G25 *etc.*, in order of increasing pore size). Because of the free hydroxyl functions, Sephadex G is strongly hydrophilic, and can be used only with water or electrolyte solutions as eluant. Several lipophilic derivatives had been prepared prior to 1970 when this work commenced. Determann prepared an acetyl derivative by reaction with acetic anhydride in benzene,[85] and Heitz prepared a urethane derivative by reaction with alkyl isocyanates in dimethyl sulphoxide.[86] However, the preferred method of attaching substituents is through an ether linkage, which confers greater chemical stability on the product.

Nyström and co-workers prepared a methylated Sephadex derivative which swelled in a variety of organic solvents and which was applied to the separation of a wide range of lipids (see refs. 83 and 87 and references therein). A hydroxypropyl derivative of Sephadex G-25 has been prepared by base-catalysed reaction with propylene oxide[88-90] and this is now available commercially as LH-20. It is "hydrophilic-lipophilic", absorbing

both water and polar organic solvents.[88] Ellingboe further effected the reaction of LH-20 with a mixture of terminal alkyl oxides (C_{11} - C_{14} chain length: "Nedox 1114") to produce a fully lipophilic hydroxyalkyl LH-20 derivative[89,90] which swells in non-polar organic solvents. This derivative can be used for the three types of system described above. Thus a gel filtration system was used for plant terpenoids[91,92] and recently a reversed-phase system was used for the separation of prostaglandins.[93] A trimethylsilyl derivative of Sephadex G has been prepared for use in straight or reversed-phase systems.[94]

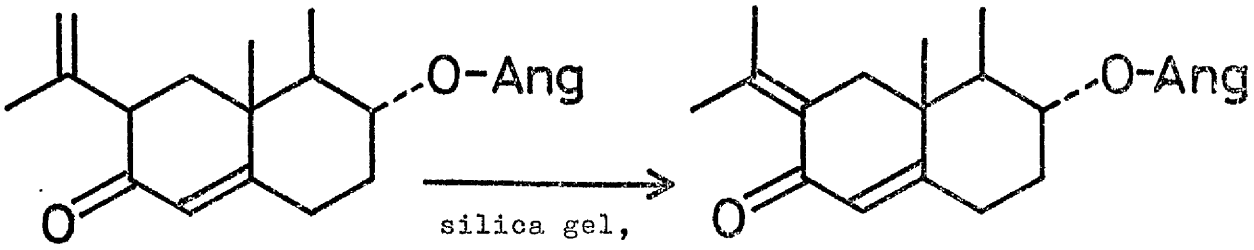
The ease of substitution of dextran gels has promoted work on the design of selective stationary phases. Ellingboe *et al.* introduced various specific groups into the sugar ring with this intent.[95] More recently, phosphatidylcholines have been separated according to the length and degree of unsaturation of the side-chain on a Sephadex-based lipophilic ion-exchange column.[96] An interesting application of a Nedox 1114 LH-20 column involved the separation of β-sitosterol and campesterol in a reversed-phase system.[97] This type of system is more sensitive to changes in the hydrocarbon skeleton than would be a straight-phase system.

Studies with the hydroxyalkyl derivative of Ellingboe have revealed advantageous properties of lipophilic gels. Mild operating conditions are obtained during chromatography. The interactions between solute and gel are generally weak and therefore this type of chromatography is well suited for labile compounds. Chromatography on active materials (silica gel, alumina, DEAE cellulose *etc.*) is known to cause rearrangements in some cases,



Germacrene-C

δ-Elemene



Petasin

Isopetasin

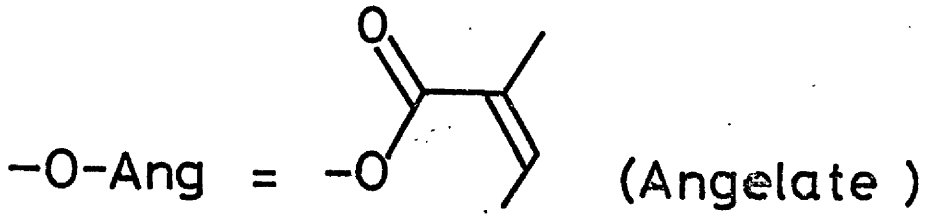


Fig.1.10 Rearrangements which occur during chromatography on active stationary phases.

for example germacrene-C to δ-elemene[98] and petasin to isopetasin[99] (Fig. 1.10). Petasin,[100] and the prostaglandins[93] are examples of sensitive compounds which have been chromatographed on a lipophilic gel. A further result of weak solute-gel interactions is that recovery of material from gel columns is usually high[92,93] and peak shapes are symmetrical.[92] As noted earlier, the capacity of soft gels is high, and even at high loads, elution profiles retain their symmetry.[92]

The potential value of lipophilic gels for the separation of lipids is apparent. The following chapter describes the preparation of derivatives of Sephadex LH-20, which, it was hoped, would be useful for lipid chromatography in general, and which would show stereoselectivity with a view to the chromatographic resolution of enantiomeric and diastereomeric compounds.

Chapter 2. EXPERIMENTAL SECTION

2.1 General Spectroscopic and Chromatographic Procedures.

Routine infrared spectroscopy was carried out using a Unicam Model SP 1000 spectrophotometer. Solution spectra were recorded on Perkin-Elmer Model 225 or 257 spectrophotometers. A Unicam SP 800 ultraviolet spectrophotometer was used in the measurement of the elution profile of racemic usnic acid (Chapter 3). Spectra were recorded in ethanol using 1 cm path-length cells. NMR spectra were recorded on a Varian T60 spectrometer. Optical rotation measurements were taken with a Perkin-Elmer Model 141 Polarimeter. A micro-capacity cell was used (volume 1 ml. path length 10 cm) with this instrument. Error in the angular rotation was of the order of $\pm 0.005^{\circ}$. Scintillation counting was done on a Philips PW 4510 Automatic Scintillation Analyser. Samples (volume 15 ml) were prepared in Packard vials using 2,5-diphenyl-oxazole (PPO, 5.5 g/l) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP, 0.1 g/l) in toluene. Melting points were recorded on the Kofler micro hot stage apparatus.

Thin-layer chromatography (TLC) was carried out on plates prepared from Merck Kieselgel-G, using 0.25 mm layers for analytical purposes and 0.5 mm layers for preparative separations. Material on plates was located using one of the following reagents:

iodine vapour or 2% ceric sulphate in 2N sulphuric acid, as general purpose reagents;

2,4-dinitrophenylhydrazine (2,4-DNP) in methanol/H_2SO_4.[101]

Bands on preparative TLC plates were located by edge development

using these reagents.

Column liquid chromatography on alumina utilised glass columns of conventional design fitted with stopcocks. A layer of acid-washed sand on cotton wool was used as a bed support. Neutral alumina (M. Woelm Eschwege, Germany) was deactivated to grade III with water prior to use.

Gas-liquid chromatography (GLC) was carried out using a Pye 104 chromatograph or on the LKB-simulating chromatograph described in Appendix 1. Glass columns, 150 cm or 300 cm (I.D. 3.5 mm) were used for these instruments respectively, and these were silanised before packing with a 5% solution of dimethyldichlorosilane (DMCS) in toluene. Packings for GLC columns, containing 1% by weight of stationary phase (10% for PEGA) were prepared using 100-120 mesh Gas Chrom Q (Applied Science Laboratories Inc.) by the method of Horning.[102] The stationary phases used were:-

OV-1 - Methyl siloxane polymer;

OV-17 and OV-25 - phenyl methyl siloxane polymers;

QF-1 - fluoroalkylsiloxane polymer;

PEGA - polyethyleneglycol adipate.

Packed columns were conditioned by treatment with bis-N,O-trimethylsilyl acetamide (BSA). The carrier gas (nitrogen) flow rate was normally adjusted to 40 ml/min. Samples, 0.5 - 2μg, were injected in 0.2 - 2μl solvent from a Hamilton micro syringe.

GLC-mass spectrometry (GLC-MS) was effected using an LKB 9000 gas chromatograph-mass spectrometer at electron energy 70eV. Line diagrams were obtained following processing of tabulated mass

spectral data on the KDF9 computer.

2.2 General Chemical Techniques.

Solvents for use in gel chromatography and in gel reactions required to be dried and distilled before use. Removal of non-volatile material from solvents was essential in order to obtain low background noise with the liquid chromatographs[83] (see para. 2.9). Drying procedures were as follows:-[103]

benzene: calcium hydride, refluxed overnight;

methanol: distillation from aluminium methoxide, followed by refluxing in air over anhydrous copper (II) sulphate;[104]

methylene chloride (CH_2Cl_2): Linde type 4A molecular sieve;

tetrahydrofuran (THF): distillation from Cu_2Cl_2 to remove peroxides, then refluxing over lithium aluminium hydride ($LiAlH_4$);

diethyl ether: by standing over sodium wire;

pyridine: refluxing over calcium hydride.

Routine solvents were of AnalaR grade (from Hopkin and Williams Ltd., Essex). Chloroform for infrared spectroscopy was dried by passage through a column packed with silica gel.

The preparation of derivatives for chromatography was carried out by the methods described below.

Methylation of carboxylic acids.

An ethereal solution of diazomethane was prepared from bis-(N-methyl-N-nitroso)-terephthalamide (Nitrosan).[105] Acids to be methylated were dissolved in ether or water[106] (for hydrophilic

substances) and treated with diazomethane until a yellow coloration persisted. The ester was then obtained by evaporation of the solvent with a stream of dry nitrogen.

Preparation of acetates.

The substrate (1 mg) was dissolved in dry pyridine (50 µl) and treated with excess acetic anhydride (50 µl). The solution was warmed at 60-80°C for 10 min and then solvent and excess reagent were evaporated under a stream of nitrogen. The product could generally be used directly for GLC without further purification.

Preparation of trimethylsilyl (TMS) ethers.

The substrate (1 mg) dissolved in dry pyridine (50 µl) was treated with hexamethyldisilazane (50 µl) and trimethylchlorosilane (10 µl). By warming at 60-80°C, the reaction was generally complete within 5 minutes. The solution was then evaporated to dryness with a stream of nitrogen and the residue triturated with ethyl acetate. Filtration through a cotton-wool plug yielded the product ready for chromatography.

Preparation of O-methyl oximes.

The substrate (1 mg) was dissolved in pyridine (50 µl) and was treated with a large excess (20:1) of methoxylamine hydrochloride as a saturated solution in pyridine. The reaction was complete in 5 min at 60-80°C. After evaporation of the solvent, trituration of the residue with ethyl acetate yielded a product which was ready for chromatography. Where necessary, the solution was washed with water to remove traces of reagents.

Preparation of 2,4-dinitrophenylhydrazones.

The substrate (100 mg) was dissolved in the minimum quantity of methanol or ethanol and treated with 0.5 ml of a solution prepared from 1g 2,4-dinitrophenylhydrazine in 30ml MeOH and 2ml sulphuric acid. The precipitated derivative was filtered, washed with ethanol and recrystallised from ethanol.

2.3 Sources of reagents and standards.

Materials were obtained from the following sources:

Sephadex G-25: Pharmacia, Uppsala, Sweden;

Deoxycholic acid, dehydrocholic acid and cyclohexene oxide: Koch-Light Laboratories Ltd., Colnbrook, Bucks;

α-Pinene and exo-2,3-oxidonorbornane: Ralph N. Emanuel Ltd., Wembley, England;

Nedox 1114: Ashland Oil and Refining Co., Minneapolis, Minn., U.S.A.;

β-(3,4-oxidocyclohexyl)-ethyltrimethoxysilane: Silar Laboratories Inc., P.O. Box 86, Watervliet, N.Y. 12189;

World Standard Pyrethrum Extract: Pyrethrum Marketing Board of Kenya (by courtesy of Dr. S.W. Head);

Human aortal lipid extract: Dr. J.D. Gilbert and Dr. A.G. Smith, Glasgow University;

Campesterol and dihydrobrassicasterol: Dr. M.J. Thompson, U.S. Department of Agriculture, Beltsville, Maryland;

Poriferasterol: Dr. G.W. Patterson, Department of Botany, University of Maryland;

5β-Cholan-7α-ol and 5β-cholan-7β-ol: Professor M. Martin-Smith;

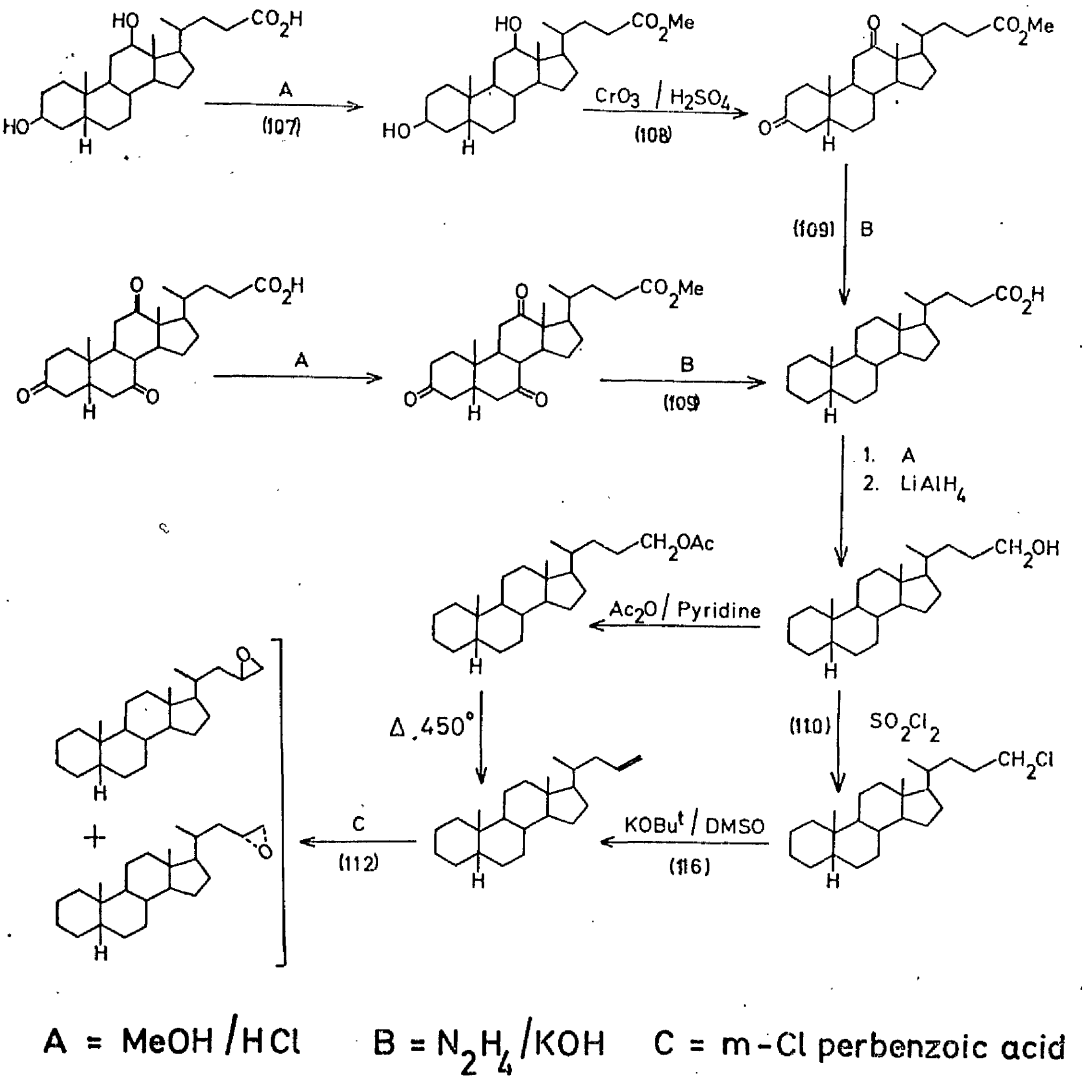


Fig.2.1 Reaction sequences for the preparation of 23,24-oxido-5β-cholane. References are quoted in parenthesis.

5-Cholestene-3β,24-(RS)-diol 3β-acetate: Dr. G.F. Woods, Organon Research Laboratories.

2.4 Synthesis of epoxides.

The preparation of three optically active epoxides was undertaken. In each case, readily available natural products were chosen as starting materials, and reactions were chosen which maintained chirality through to the epoxide.

2.4.1 23,24-Oxido-5β-cholane.

This epoxide was prepared from both deoxycholic acid and dehydrocholic acid. The reaction sequences are summarised in Fig. 2.1. Brief experimental details follow for reactions not annotated with literature references.

Methylation of bile acids.

The acid was dissolved in a 3% methanolic solution of hydrogen chloride[113] and the mixture boiled under reflux until reaction was complete as indicated by TLC (usually 30 min). Volatiles were removed by vacuum distillation, and the solid product was recrystallised from methanol.

Reduction of methyl 5β-cholanoate to 5β-cholan-24-ol.

A suspension of lithium aluminium hydride (2 equivalents) was stirred for 1 h at room temperature in dry ether under a nitrogen atmosphere, and methyl 5β-cholanoate (1 equivalent) was added slowly in ether solution. The reaction was almost complete (>90%) after a further 1 h. Excess hydride was destroyed by the cautious addition of ethyl acetate. Water was added and 5β-cholan-24-ol was recovered by ether extraction.

TABLE 2.1 Chromatographic data for intermediates in the synthesis of 23,24- oxidocholane.

Retention indices at T°C stated (in brackets).

Compound	1% SE 30	1% OV-1	1% OV-17
Deoxycholic acid tris-TMS derivative	3261(223°)		
Methyl deoxycholate	3200(223°)		3630(223°)
Bis-TMS-methyl deoxycholate	3155(223°)		3330(223°)
Methyl dehydrodeoxycholate	3235(241°)		
Cholanic acid TMS derivative	2946(241°)		3160(241°)
Methyl cholanoate	2822(225°)		3107(225°)
5β-Cholan-24-ol	2800(225°)	2795(225°)	3280(220°)
5β-Cholan-24-ol TMS ether	2853(225°)	2833(220°)	
5β-Cholan-24-ol acetate	2908(225°)		3171
24-Chloro-5β-cholane	2780(220°)	2770(220°)	3030(225°)
5β-Chol-23-ene		2490(225°)	
24-Butoxy-5β-cholane		2870(225°)	
23,24-Oxido-5β-cholane		2718(225°)	
Methyl lithocholate		3035(225°)	

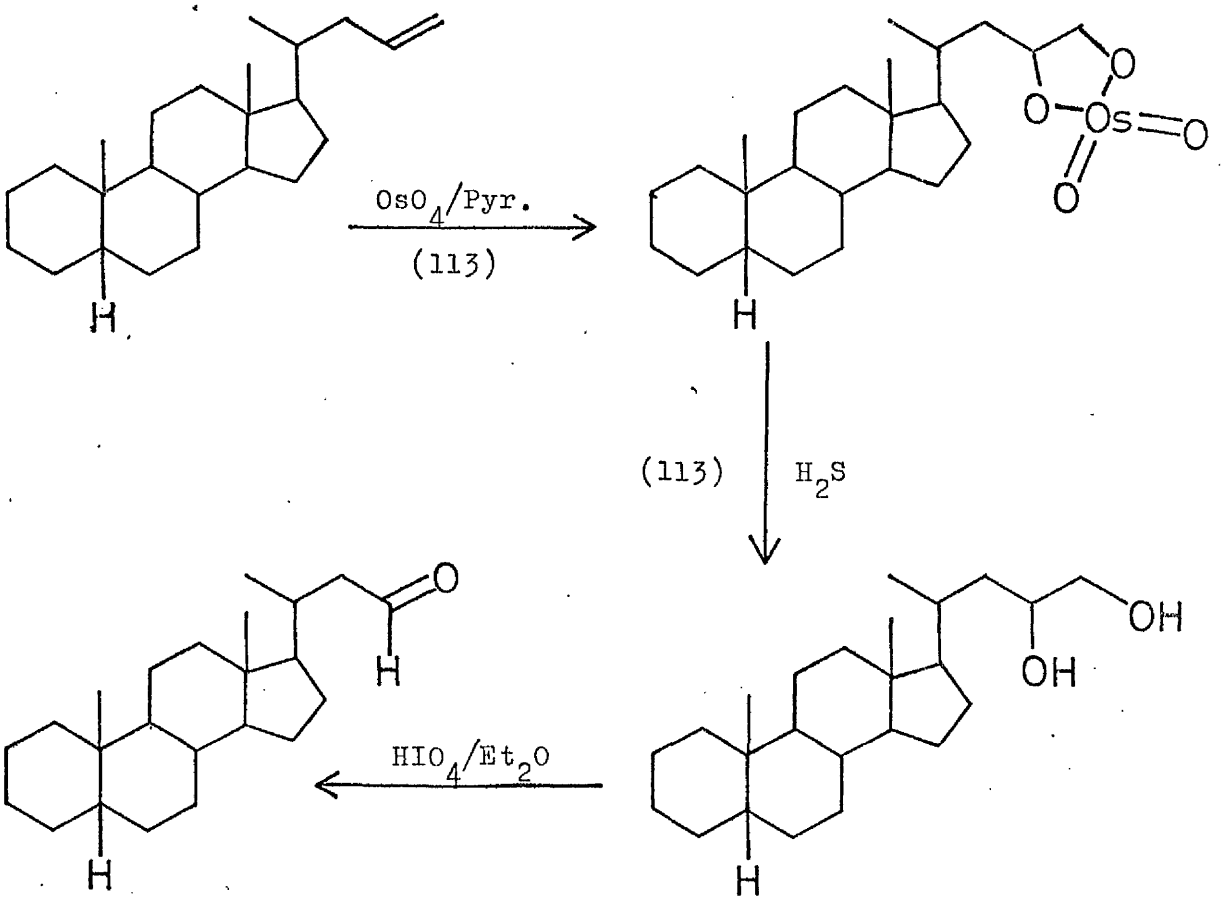
A sample recrystallised from ethyl acetate had m.p. 131-131.5° (recorded[110] m.p. 130.5-132.5°).

Preparation of 5β-chol-23-ene.

Two methods were used, starting from 5β-cholan-24-ol.

(i) The alcohol was converted to the chloride by reaction with sulphuryl chloride[110] and the chloride treated with potassium t-butoxide in dimethyl sulphoxide.[111] In agreement with the previous work by Wood and Chang,[111] this gave predominantly the olefin, with some 24-t-butoxy-5β-cholane (17%). The latter was further characterised by GC-MS: I_{OV-1} 2870; $M^{+\cdot}$ 402, major fragment ions at m/e 387, 346, 345, 328, 217, 149, 109, 95, 57. The bulk of the olefin (I_{OV-1} 2490) was not purified before epoxidation as the t-butyl ether was found not to interfere in any of the subsequent reactions.

(ii) Vapour-phase pyrolysis of 5β-cholan-24-yl acetate gave an oil containing 83% by weight of the olefin (estimated by GLC). A solution of the acetate in light petroleum (b.p. 40-60°) was added in a slow nitrogen stream to a vertical silica tube packed with glass wool and maintained at 450°C by an electric oven. The product emerged from the bottom of the tube as an aerosol which was collected in a cooled trap. Purification of the olefin was effected by column chromatography on alumina, followed by recrystallisation from methanol; the product melted over the range 87.5-95° (recorded[111] m.p. 99-100.4°C). The presence of the terminal double bond was confirmed by NMR (60 MHz in $CDCl_3$): multiplets corresponding to 2 vinyl protons at 5.0 p.p.m. and 1 vinyl proton at 5.7 p.p.m. The structure of



Mol.Wt. = 330
(O-Methyl oxime = 359)

Fig.2.2 Oxidative cleavage of the olefinic material obtained by pyrolysis of 5β-cholan-24-ol acetate. Mass spectral data were obtained using the LKB 9000 instrument.

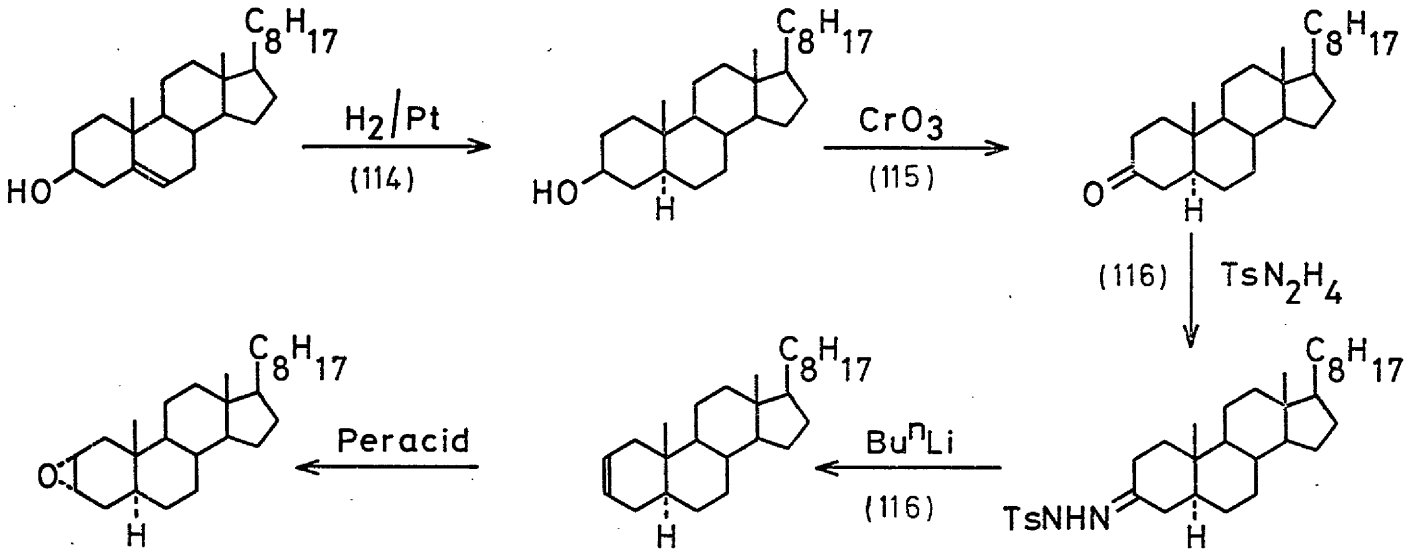


Fig.2.3 Reaction sequence for the preparation of 2α,3α-oxido-5α-cholestane from cholesterol.

the olefin was confirmed by oxidative cleavage of the double bond with osmium tetroxide[112] and periodic acid (Fig. 2.2). Analysis of the product and of its methyl oxime by GC-MS indicated a molecular weight of 330 for the free carbonyl compound and 359 for the oxime. These values correspond to products arising from 5β-chol-23-ene. GLC of the cleavage products indicated that this olefin constituted over 90% of the pyrolysis product.

2.4.2 2α,3α-Oxido-5α-cholestane.

This oxide was prepared from cholesterol by the reaction sequence shown in Fig. 2.3. Literature references are indicated in parenthesis. The following physical properties and yields were recorded.

Cholesterol starting material.

100 g crude cholesterol recrystallised from MeOH (1750 ml) gave 76.5 g of material mp. 145-147° (Lit.[114] mp. 148.6-149.8°).

5α-Cholestan-3β-ol.

From 75 g cholesterol, 60 g recrystallised (EtOH) product (80%) were obtained, mp. 140.5-141° (Lit.[114] mp. 139-141°).

5α-Cholestan-3-one.

5α-Cholestan-3β-ol (59 g) yielded 51.5 g (87%) of cholestanone which on recrystallisation from EtOH (200 ml) gave 49.8 g material mp. 127.5-129° (Lit.[115] mp. 129-130°).

5α-Cholestan-3-one N-tosyl hydrazone.

5α-Cholestanone (20 g) yielded 20 g (80%) of the hydrazone mp. 165-168° (Lit.[116] 168-170°). On the small scale, 1 g ketone gave 1.4 g hydrazone (quantitative conversion).

5α-Cholest-2-ene.

5α-Cholestan-3-one tosyl hydrazone (18 g) gave 13 g of an oil which was analysed by chromatography.

TLC (developed $CHCl_3$): the product contained materials of R_f value 0.9, 0.6, 0.5. Standards of cholest-2-ene and 5α-cholestan-3-one tosyl hydrazone had R_f values of 0.9 and 0.5 respectively. GLC analysis (OV-1, 250°C) indicated one major GLC-volatile peak (95% of total). GLC-MS indicated an M^+ of 370 and a major ion at 316, corresponding to loss of C_4H_6.

The oil could not be crystallised. The product was purified by chromatography on alumina, giving 8.5 g material which was pure on TLC and GLC. I.R. spectroscopy indicated the presence of an olefinic double band (absorptions at 3020-3015, 1650, 775, and 670 cm^{-1}).

Epoxidation of 5α-Cholest-2-ene.

The olefin (8 g, 0.022 moles) was dissolved in dry CH_2Cl_2 (25 ml) and the solution was cooled to 0°C. m-Chloroperbenzoic acid (4.2 g, 0.024 mole) was added in portions to the stirred solution and the resulting suspension stirred at room temperature for 3 h. The solution was filtered and diluted (100 ml CH_2Cl_2) to facilitate handling. Excess peracid was destroyed with sodium bisulphite (1 g in 20 ml H_2O). The solution was washed consecutively with aqueous sodium bicarbonate and water, dried and evaporated to yield the product (8.2 g, 89%). A sample recrystallised twice from Et_2O-MeOH had mp. 95-100°C (Lit.,[117] 2α,3α-oxide mp. 104-105°C, 2β,3β oxide mp. 91°C). The oxide was presumed to be mostly the α-isomer.[117] Chromatographic analysis of the crude

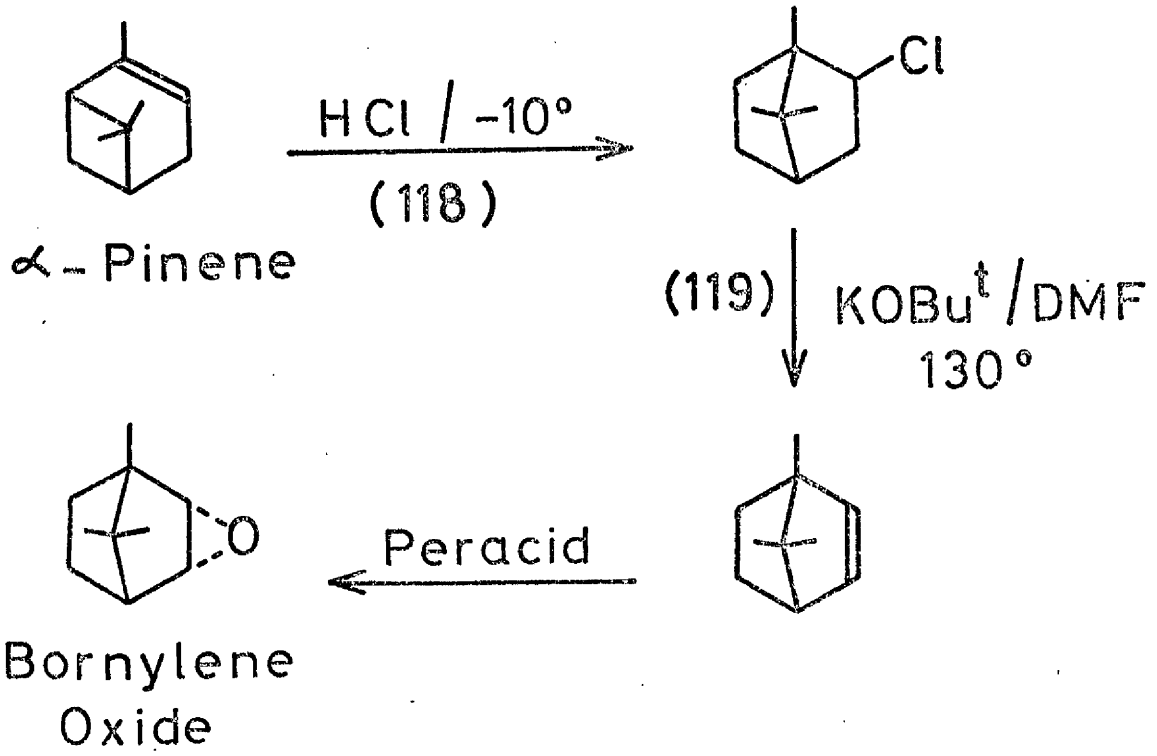


Fig.2.4 Preparation of (+)-endo-2,3-oxidobornane.

product gave the following data:-

TLC: (developed benzene): R_f values 0.9, 0.4, 0.3 (main spot).

Standards: 5α-cholest-2-ene, 0.9; 5α-cholestan-3-one, 0.5.

GLC: 1 major peak $I^{260^\circ}_{SE30}$ 3045 (65% of total) and 6 minor peaks.

The NMR spectrum showed an unresolved multiplet at 6.8-7.0 ppm and a partly-resolved doublet at 7.2-7.3 ppm.

2.4.3 (+)Endo-2,3-oxidobornane.

The synthesis from (+)-α-pinene was by the reaction sequence shown in Fig. 2.4. This follows the method of Borowiecki et al.,[119] with the exception that m-chloroperbenzoic acid was used for the epoxidation of bornylene instead of p-nitroperbenzoic acid. The following data were recorded.

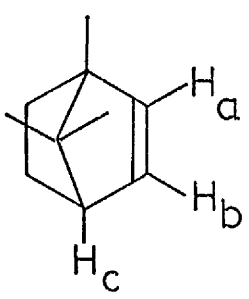
Bornylene.

This was obtained in approximately 70% yield from bornyl chloride, following purification by fractional distillation through a 40 cm. Vigreux column.

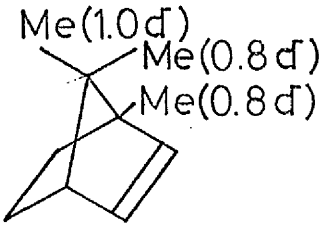
TLC: (developed 60-80° B.P. petroleum ether) two spots were visible, R_f values 0.85 and 0.75. Bornyl chloride had an R_f value of 0.5 and an impurity in this material had an R_f value of 0.75.

I.R.: Absorptions at 3060, 1620, 1370, 1360, and 715 cm^{-1}. The latter is characteristic of bornylene.[119] Two impurity bands (3150w, 2720w) were observed.

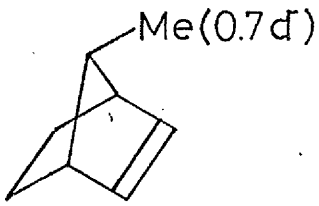
NMR: In $CDCl_3$ solution, the following signals were observed. 5.9 ppm (1 H_b), 5.6 ppm (1 H_a), 2.3 ppm (1 H_c) which were assigned as follows (I):-[120]



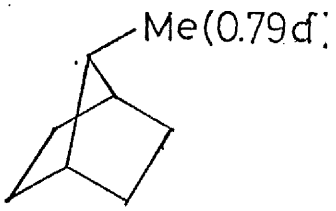
I



II



III



IV

In addition, signals for the methyl groups were observed and assigned as indicated (II) by analogy with compounds III and IV.[120]

2,3-Oxidobornane.

The crude oxide was obtained in 76% yield from bornylene. Purification by sublimation or recrystallisation gave poor recoveries (50-60%) and generally these processes were inefficient. The following data were recorded.

TLC: (developed in 60-80° B.P. petroleum ether). The oxide had an R_f of 0.25. Impurities appeared at 0.8 and 0.85, of which the latter corresponded to bornylene.

I.R.: The characteristic band[119] at 865 cm^{-1} was observed. Small amounts of bornylene were present, indicated by the 715 cm^{-1} band.

NMR: The oxide protons appeared at 3.53 ppm (triplet) and 3.23 ppm (doublet).

2.5 Size Fractionation of Sephadex G-25.

High resolution columns required gel particles of a narrow size range. It has been shown[83,121-123] that significant improvements in theoretical plate heights are obtained with particles of uniform diameter. The height equivalent to a theoretical plate

(HETP) tends to decrease also when the particle size is reduced. The partition process between mobile and stationary phases is more rapid with small particles ($<30\mu$), allowing higher flow rates to be used while still maintaining resolution. Resistance to solvent flow through a bed packed with small particles is high and requires the use of higher operating pressures than are needed in classical liquid chromatography. Sjövall found[83] that unfractionated fine Sephadex G-25 (40-80μ diameter in the dry state), superfine (10-40μ) and fractionated superfine (17-23μ) had under similar operating conditions HETP values of 0.60, 0.25 and 0.12 mm respectively.

The value of fractionation is apparent, although whether the considerable time and effort required to effect the process is worthwhile must depend on the ultimate application of the gel material. For easy chromatographic separations (α values ≥ 1.5), removal of fines, for example by decantation of a suspension of the material in a suitable liquid, followed by crude fractionation with sieves provides particles of adequate quality. Sieving produces relatively large size-range fractions and contamination often arises from aggregation of small particles and from fragmentation of beads. The present work was concerned with separations where α was low (≤ 1.3). It was considered worthwhile to grade further the Sephadex beads. Two elutriation methods were used.

(a) Continuous-flow differential sedimentation method (aqueous).

The principle of this method was described by Hamilton.[124] Sephadex G-25 (100 g fine grade) was equilibrated[84] for 3 h in water containing 1% sodium azide as bacteriostat. The gel was

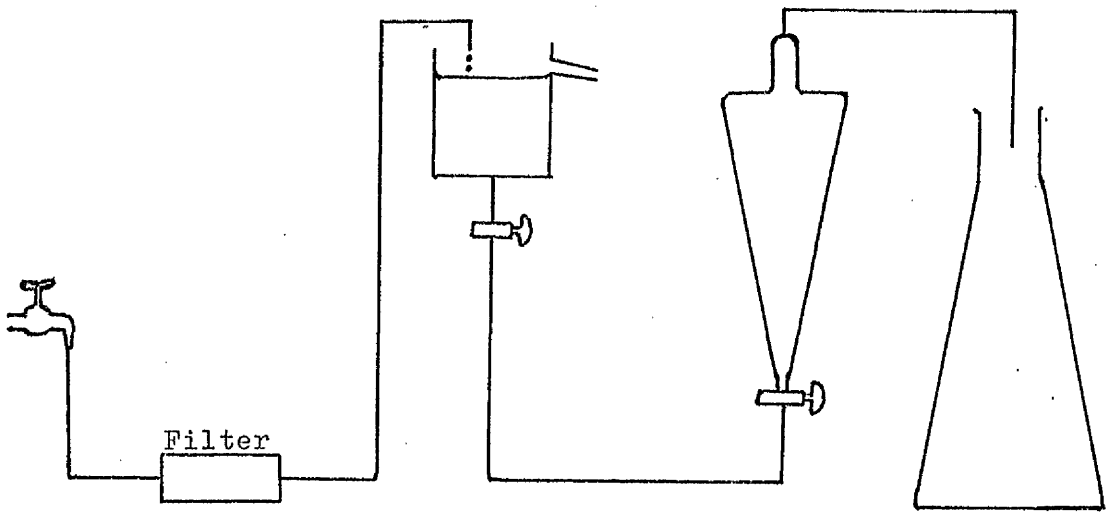


Fig.2.5 Apparatus for the size-fractionation of Sephadex G-25 by the aqueous sedimentation method of Hamilton.

Fig.2.6. Apparatus for the size-fractionation of Sephadex by elutriation in a gas stream.

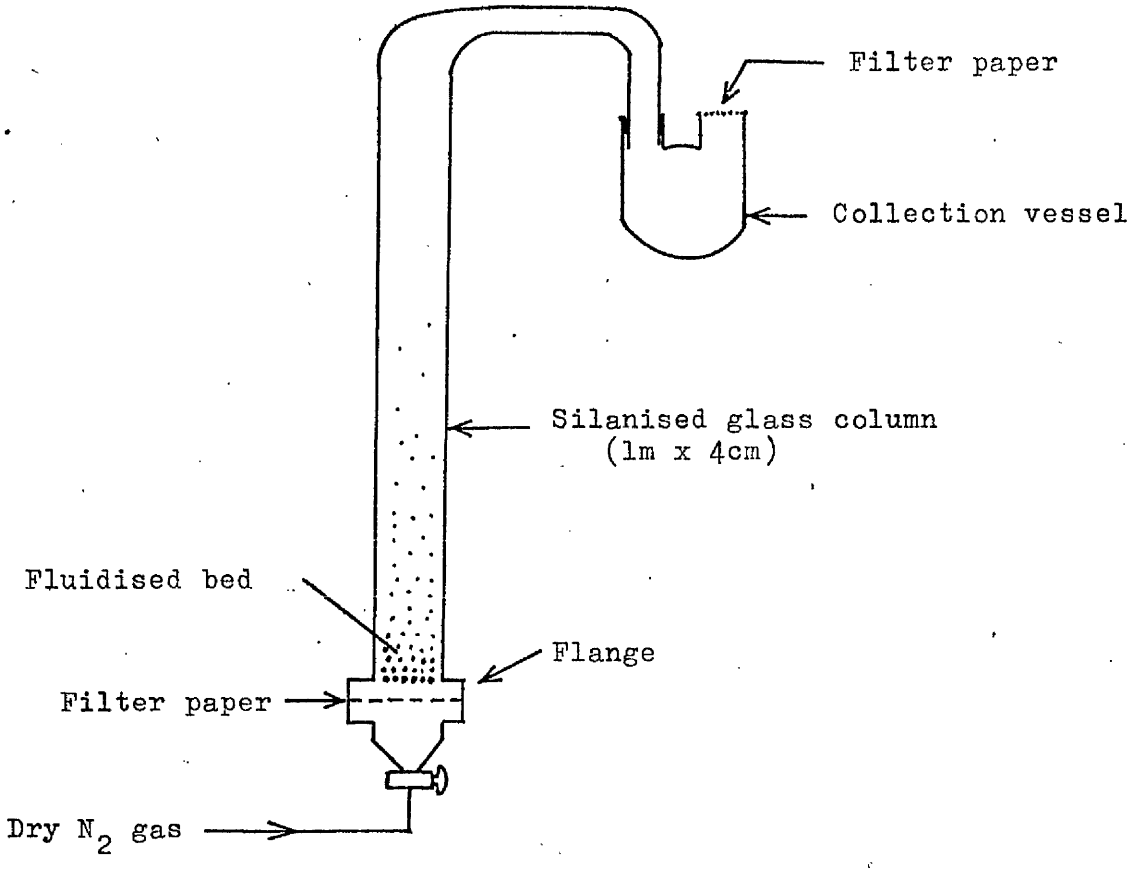


TABLE 2.2 Fractions obtained by water-sedimentation of Sephadex G-25 (105 g., Fine Grade).

Flow rate ml/min.	Size range of dry gel beads (μ).	Weight of fraction (g)
100	Fines	Discarded
160	20-30	4.4
250	30-40	13
350	40-45	17
450	45-50	14
580	50-60	16
(Residue)	>60	40

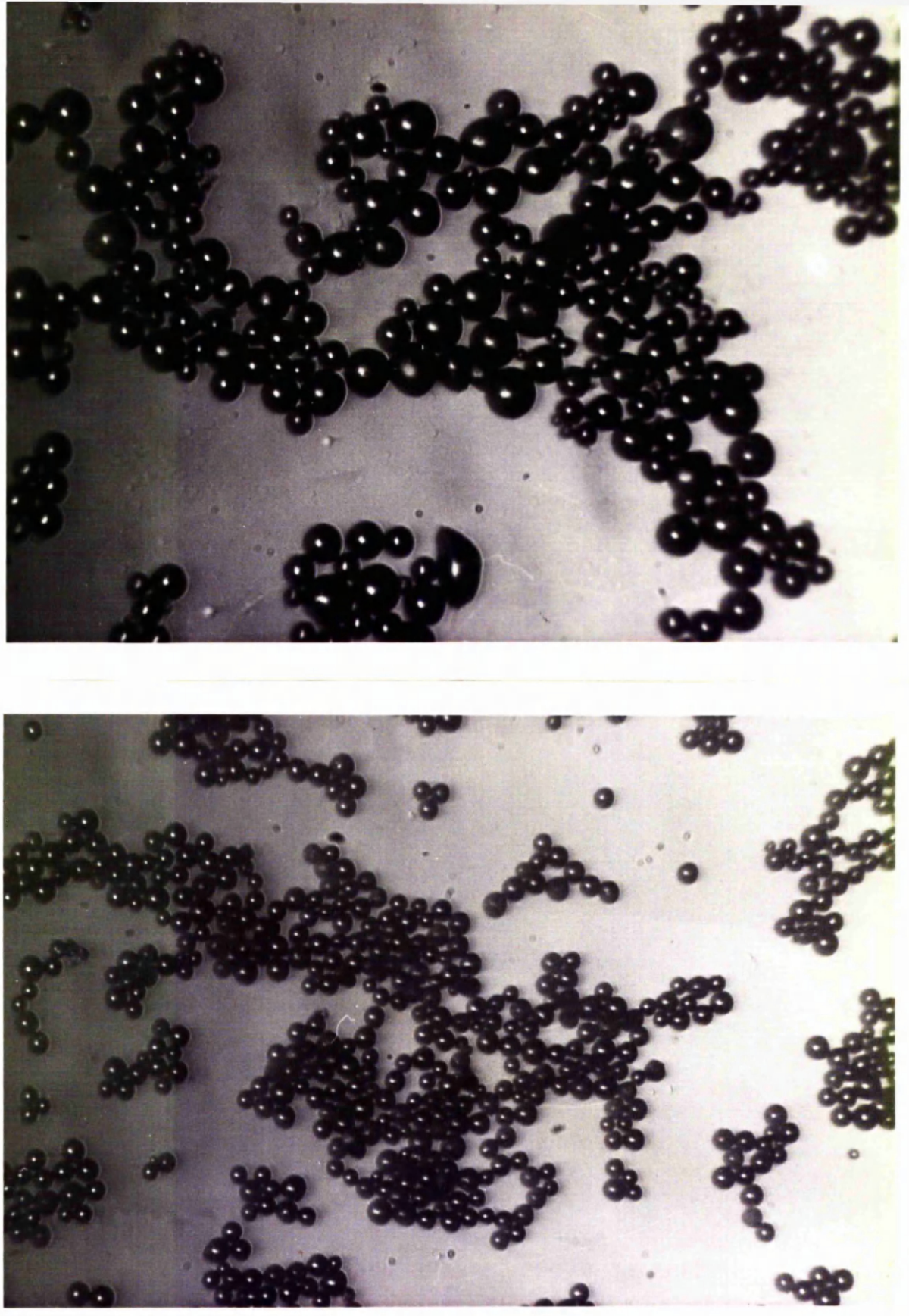


Fig.2.7 An illustration of particle fractionation. The micrographs show Sephadex beads (G-25, superfine 10-40 μ) before fractionation (upper) and after (lower). Elutriation in a gas stream was used to separate the particles.

then transferred to a 5l conical separating funnel (Fig. 2.5). The lower end of the funnel was connected to a water supply through a filter (20μ mesh, from Filinox Filters, P.O. Box 3, Chard, Somerset) which removed algae and other sediment. Flow rates were controlled by Quickfit Rotaflo valves and, at lower flow rates, by drawing water from a reservoir which could be adjusted to maintain a constant hydrostatic pressure. A vertical velocity gradient was set up in the separating funnel: stepwise increases in the flow displaced successive fractions into the collecting vessel. Following decantation of the supernatant, the collected gel was washed with distilled water, dried and weighed. Table 2.2 shows the particle diameters of fractions (estimated using a microscope graticule) versus flow rate.

(b) Elutriation in a nitrogen stream.

This method, adapted from a description by Fischer,[125] makes use of the different Stokes' velocities in a gas of particles of different diameter. Fig. 2.6 illustrates the apparatus. A stream of dry nitrogen from a cylinder was passed through the Sephadex beads (25 g), (which were supported on filter paper) forming a fluidised bed. After removal of fines, the flow rate was increased in stages and fractions were carried over into the trap. This method was successful for the earlier fractions, but later material was contaminated with particles of smaller diameter (Fig. 2.7).

2.6 Preparation of modified gels.

This section deals with initial and subsequent reactions of

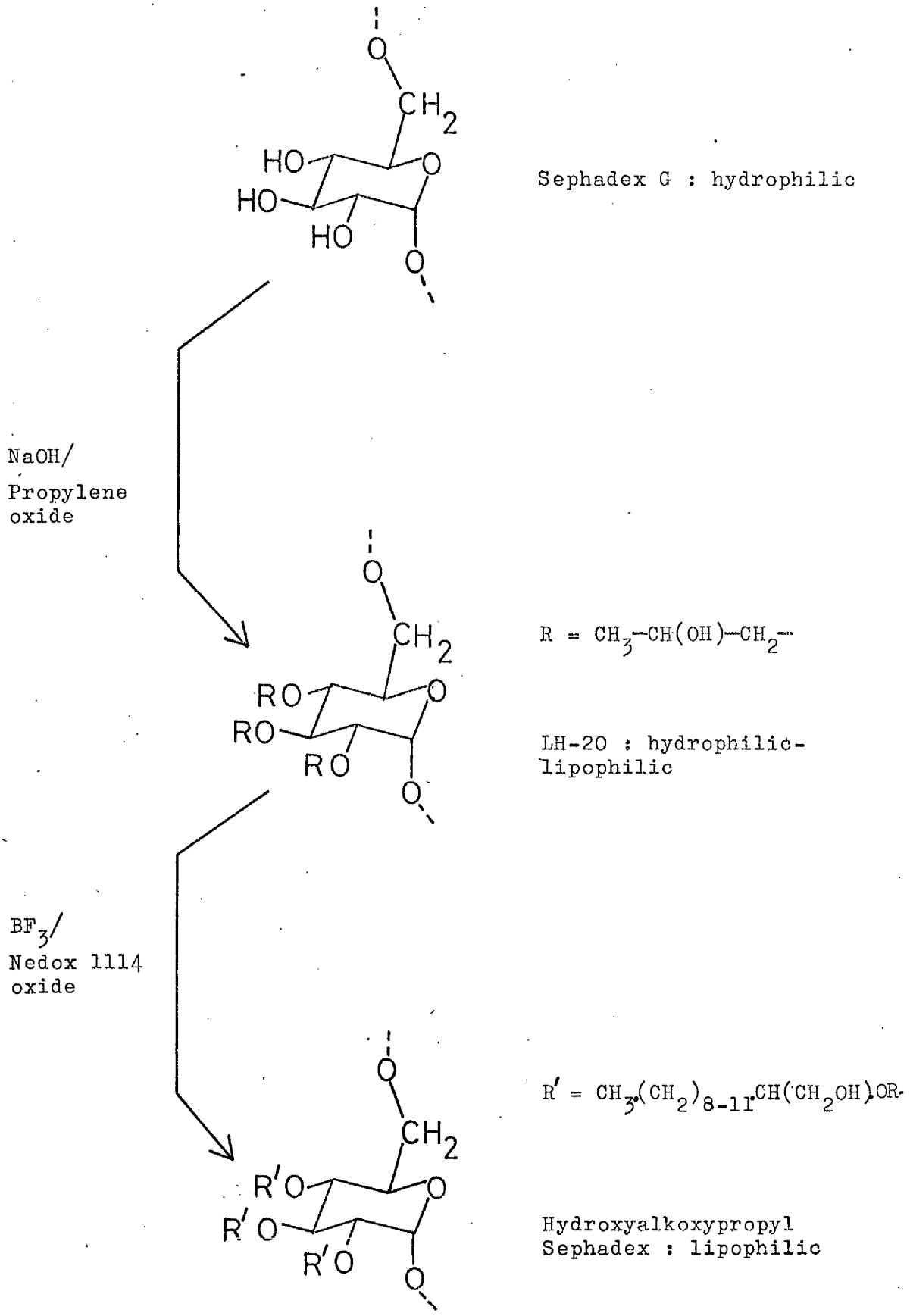


Fig.2.8 Reaction sequence for the preparation of hydroxyalkyl LH-20.

Sephadex G-25 in the preparation of hydrophobic gels. The unsubstituted dextran polymer is very hydrophilic and the problem of finding a good reaction medium, in which the gel will swell and at the same time in which the lipophilic reagent will dissolve, is circumvented if the gel is first converted to a hydrophilic-lipophilic derivative.[87-90] Ellingboe and co-workers used a β-hydroxypropyl derivative of the dextran as an intermediate in the preparation of hydroxyalkoxy Sephadex (Fig. 2.8). Hydroxypropyl Sephadex prepared from G-25 is marketed under the name LH-20. Analogues of LH-20 can be prepared from other G types,[89] leading to derivatives with various degrees of cross-linking between the dextran chains in the polymer matrix.

The fractions obtained from the sedimentation processes were converted to LH-20 type derivatives by the following method.[89,90]

Preparation of hydroxypropyl Sephadex.

Sephadex G-25 was gently stirred at RT with 4% aqueous NaOH for 2 h. Mechanical stirring was used in this reaction and in all subsequent gel reactions as magnetic stirring caused fracture of the Sephadex beads. Stirring rods were constructed from glass rod in preference to commercial link-type stirrers, and care was taken to avoid abrasion of the gel between the glass surfaces. After excess NaOH was filtered off, the swollen gel was suspended in propylene oxide (35 ml per gram dry gel) and was refluxed with stirring overnight. The gel was filtered, washed successively with acetone, distilled water (until free of alkali), acetone again and finally petroleum ether (40-60° B.P. fraction). The gel was dried firstly on the filter pad and then in a vacuum oven at 60°C.

The dry weight of the product was approximately twice that of the starting material, corresponding to 100% substitution of the hydroxyl groups on the dextran.

Ellingboe and co-workers found that further substitution reactions of LH-20 with Nedox 1114 n-alkyl epoxide proceeded readily with an acid catalyst,[89,90,126] boron trifluoride etherate. In the alkali-catalysed substitution reaction above, and in the BF_3-catalysed reaction of LH-20 with Nedox 1114, large excesses of oxide were used. Ring opening of epoxides by solvolysis or to give rearrangement products results in competing side reactions which lower the efficiency of the substitution process. In view of the length of the synthesis of 23,24-oxido-5β-cholane, relatively small amounts of this oxide were available for the preparation of a modified gel. The experiments described below were carried out in order to reduce the amounts of oxide required, approximately, to stoichiometric amounts. The method of preparation of the gel was developed with the aim of introducing one cholanyl substituent into each sugar residue in the dextran, corresponding to a hydroxy-5β-cholanyl content of 50.5% by weight, analogous to the Nedox gel prepared by Ellingboe, which contained 50% by weight of hydroxy-alkyl substituents. Trial reactions were carried out with approximately stoichiometric quantities of Nedox 1114 oxide, varying the conditions used. The oxide mixture was analysed by GLC and found to contain approximately equimolar amounts of C_{11}, C_{12}, C_{13} and C_{14} epoxides. An average molecular weight of 191 was used in computing amounts of material for reactions.

TABLE 2.3 Tabulation of experiments carried out.

No.	LH-20(g)	LH-20(equivalents)*	CH_2Cl_2 (ml)	Nedox (g)	Nedox(equivalents)	BF_3 (ml)	BF_3 (equiv.)	Period before adding Nedox	% Uptake of Nedox
1	0.507	4.45×10^{-3}	5	0.900	5.00×10^{-3}	0.20	1.59×10^{-3}	20 min	21
2	0.504	4.45×10^{-3}	5	0.900	5.00×10^{-3}	0.63	5.00×10^{-3}	10 min	0 (Gel hydrolysed)
3	0.503	4.45×10^{-3}	5	0.300	1.67×10^{-3}	0.21	1.67×10^{-3}	20 min	0
4	0.996	8.91×10^{-3}	10	0.540	3.00×10^{-3}	0.40	3.30×10^{-3}	30 min	45
5	1.00	8.91×10^{-3}	10	0.540	3.00×10^{-3}	0.10	0.80×10^{-3}	15 min	70
6	0.992	8.91×10^{-3}	10	0.540	3.00×10^{-3}	0.02	0.16×10^{-3}	10 min	65

* Here regarded as a tribasic acid.

Examination of the reaction between LH-20 and Nedox 1114.

General Method: Sephadex LH-20 gel was dried thoroughly by washing with acetone several times and, following filtration on a glass-sintered funnel, by warming under vacuum, overnight. Because of the tendency of the gel to adhere firmly to glass surfaces, all apparatus used for conducting the substitution reaction was silanised beforehand. 0.5 gram of LH-20 was weighed accurately into a two-necked flask which was then fitted with a mechanical stirrer and pressure equilibrating dropping funnel and was allowed to equilibrate overnight in 5 ml dry methylene chloride. During this period the suspension was not stirred and all glass connections were sealed with Parafilm to exclude moisture. BF_3 etherate was then pipetted into the gently stirred gel and after a measured time the epoxide, in dry methylene chloride, was added in a dropwise manner to the flask. After reaction had occurred (9 h approximately as indicated by analytical TLC), the gel was filtered on a weighed glass sinter funnel, washed with methylene chloride and acetone, and then dried under vacuum at room temperature. Weighing of the gel was carried out as quickly as possible as water uptake from the air was rapid.

Table 2.3 summarises the experiments carried out. The optimum amount of BF_3 etherate was 0.1 ml per g of dry gel, agreeing with the findings of Ellingboe *et al.*[90] 70% by weight incorporation of the Nedox 1114 was the most efficient substitution obtained (Expt. 5, Table 2.3). The conditions in this experiment

TABLE 2.4 GLC analysis of supernatant from the reaction of LH-20 with 23,24-oxido-5β-cholane.

Retention Index I^{225}_{OV-1}	%	Composition
2480	0.8	Unknown
2715	0.9	Unknown
2870	97.3	24-Butoxy-5β-cholane
2870	Standard	24-Butoxy-5β-cholane

were used for the preparation of the cholanyl substituted gel.

Preparation of hydroxy-5β-cholanyl LH-20.

A sample of gel (4.3 g) was placed in a flask fitted with a mechanical stirrer and pressure-equilibrating dropping funnel. Dry CH_2Cl_2 (40 ml) was added, and the gel was equilibrated by gentle stirring in the solvent overnight. BF_3 etherate (0.43 ml) was added and stirring continued for 10 mins. 23,24-Oxido-5β-cholane (3.5 g) in dry CH_2Cl_2 (25 ml) was added during 30 min and the suspension stirred for 4 h. After filtering, washing with CH_2Cl_2, methanol and acetone and drying overnight under vacuum, the gel weighed 7.2 g, representing an uptake of 64% by weight of the oxide. This corresponded to 0.655 cholane residues per sugar residue. The steroidal material in the supernatant was shown by GLC to comprise over 97% of 24-t-butoxy-5β-cholane (Table 2.4).

Preparation of lipophilic gels by reaction of LH-20 with alicyclic oxides.

The successful reaction of terminal olefin oxides with LH-20 prompted the investigation of substitutions using alicyclic oxides. These reactions were potentially more difficult due to steric hindrance but the ability to use such oxides would greatly extend the range of possible substituents in lipophilic gels. The following paragraphs describe the preparation of hydroxyalicyclic derivatives of LH-20. Cyclohexene oxide was used initially as a model compound for the reaction of more complex alicyclic olefin oxides.

TABLE 2.5 Summary of experimental details of gel reactions.

Oxide	g.oxide	g.LH-20	No. of reactions	% Substitution by weight after 1st reaction	2nd reaction	3rd reaction	Final % substitution by weight.
2α,3α-Oxido-5 α-cholestane	3.0	2.6	3	16.1	31.6	37.5	37.5
β-(3,4-Oxidocyclohexyl)-ethyltrimethoxysilane	5 ml	2.5	2	32.4	34.0	-	34.0
Exo-2,3-oxidonorbornane	2.0	1.9	2	16.0	20.2	-	20.2
Endo-2,3-oxidobornane	2.1	1.5	1	7.0	-	-	7.0

Preparation of hydroxycyclohexyl LH-20.

Method 1: Stoichiometric amounts of cyclohexene oxide.

LH-20 (1.15 g, 0.0034 mole) was equilibrated with 10 ml CH_2Cl_2 for 3 h at room temperature. BF_3 etherate (0.11 ml) was added to the stirred suspension and, after 10 min, cyclohexene oxide (1.00 g, 0.0102 mole) in dry CH_2Cl_2 (10 ml) was added over 15 min. The suspension was stirred overnight at room temperature and then the product was filtered, washed consecutively with CH_2Cl_2 and acetone and dried under vacuum. The product weighed 1.48 g, representing an increase in weight of 0.28 g, and a content of hydroxycyclohexyl residues of 19% by weight or 0.83 residues per sugar ring.

Method 2: Excess cyclohexene oxide.

LH-20 (2.0 g) was suspended in dry CH_2Cl_2 (6 ml) for 1 h. BF_3 etherate (0.25 ml) was added and the suspension left to equilibrate for 15 min. The reaction flask was transferred to an ultrasonic tank and cyclohexene oxide (5 ml) in dry CH_2Cl_2 (5 ml) was added during 10 min with gentle mixing. The flask was removed from the tank and left overnight at room temperature. The gel was filtered, washed with CH_2Cl_2, ethanol and acetone and dried under vacuum. It then amounted to 3.2 g, the increase of 1.2 g corresponding to 68.5% substitution of free hydroxyl groups, i.e. two hydroxycyclohexyl residues per sugar ring.

Further hydroxyalicyclic derivatives of LH-20.

The preparation of four novel gels is described below. Table 5 summarises the details of experiments carried out. Alicyclic oxides were found to undergo rearrangement reactions and

polymerisations more readily than the alkyl oxides previously examined. The by-products have been briefly examined and characterised. Because of the inefficient incorporation of substrate, reactions were repeated in an attempt to increase the degree of substitution of the gel. In agreement with the findings of Ellingboe et al.,[90] this met with some success.

Reaction of LH-20 with 2α,3α-oxido-5α-cholestane.

The method used followed that for the reaction of LH-20 with 23,24-oxido-5β-cholane. The product from initial reaction contained 16.1% by weight of hydroxycholestanyl residues. Repetition of the substitution reaction increased this to 31.6% by weight, and a third reaction (with 1.2 g of oxide instead of 3 g) produced a gel containing 37.5% by weight of substituent. This corresponds to 0.5 hydroxycholestanyl residues per glucose ring in the dextran. On average, only 20% of the oxide was incorporated in any one step. Evaporation of the supernatant liquid yielded a pale brown solid. The following characteristics were observed:-

TLC: Two spots, R_f values 0.85 and 0.93 standards: 5α-cholest-2-ene, 0.85; 2α,3α-oxido-5α-cholestane, 0.30; 5α-cholestan-3-one, 0.12.

GLC: Conditions used were: 5 m 1% OV-1 at 260°C. No peaks were observed for the reaction product up to I value 3500 (I^{260}_{OV-1} for the oxide was 3045).

IR: Broad bands, absorption at 1090 cm^{-1}.

NMR: Characteristic steroidal pattern below 2 ppm. Resolution much lower than for the oxide, and some ether proton absorption at 3 - 4 ppm, but very broad and poorly resolved.

The material could not be crystallised, and, on the basis of the characteristics noted, was concluded to be an ether-linked polymer.

Reaction of LH-20 with β-(3,4-oxidocyclohexyl)-ethyltrimethoxy-silane.

The procedure used was as described for the reaction of cyclohexene oxide with LH-20 (Method 2, p. 70 above). The initial product contained 32.4% by weight of substituent. On repeating the reaction, this was increased only to 34.0%, which was assumed to be the maximum substitution attainable under these conditions. This corresponds to 0.7 substituent residues per sugar ring of the dextran. Trimethoxysilylethyl-hydroxycyclohexyl LH-20 will henceforth be denoted as TMSE-hydroxycyclohexyl LH-20.

The supernatant from this reaction yielded on evaporation a clear glassy material, polymeric in nature, whose exact composition was not determined.

Reaction of LH-20 with exo-2,3-oxidonorbornane.

The reaction was carried out using the method described earlier for the preparation of hydroxy-5β-cholanyl LH-20. On repeating the substitution reaction, the degree of substitution was increased from 16.0 to 20.2% by wt. This was assumed to be the maximum for the experimental conditions used. I.R. characterisation

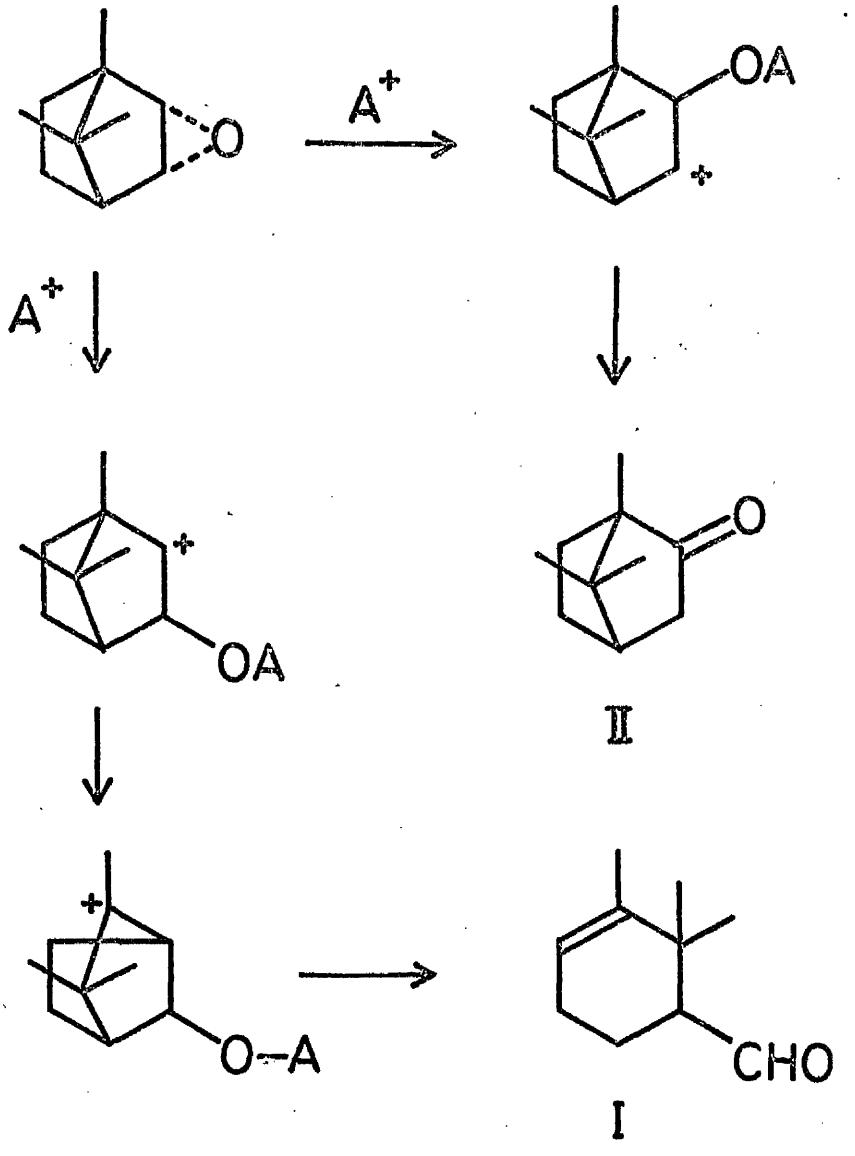


Fig.2.9 Acid-catalysed rearrangement of endo-2,3-oxidobornane. A^+ represents a Lewis acid, for example, BF_3 etherate. Product I is 4-formyl—2,3,3-trimethyl-1-cyclohexene; product II is camphor.

of the reaction by-products indicated that these were carbonyl-containing compounds, primarily ketonic (1740 cm^{-1} absorption).

Reaction of LH-20 with endo-2,3-oxidobornane.

The method used for this reaction also followed the procedure for the LH-20/cholanyl oxide reaction. Incorporation of the oxide into the gel was very low (7% by weight of substituent). The reaction could not be repeated owing to lack of oxide. The by-products of the reaction were examined by infrared spectroscopy. Two carbonyl adsorptions were observed, at 1720 and 1740 (3 h). These were presumed to be the aldehyde (I) and ketone (II) observed by Borowiecki *et al.* following a silver oxide-catalysed rearrangement of bornylene oxide,[119] for which the reaction sequence is illustrated in Fig. 2.9.

Base-catalysed substitution reactions of Sephadex.

Some experimental investigations were carried out on the possibility of using basic conditions for the substitution process. The tendency for rearrangement of an oxide during the reaction should be reduced, although the problem of solvolysis would be more important. A further possible advantage arises from work reported by Ellingboe,[95] concerning the preparation of substituted Sephadex gels, using a chlorohydroxypropyl derivative of LH-20 as an intermediate. The chlorine atoms of the substituent moieties were replaceable by reaction with basic compounds, for example amines. Reactions between alkoxides and the chlorinated Sephadex derivative would preclude the time-consuming preparation of epoxides, while still yielding derivatives in which the lipid substituents are bonded through the stable ether linkage.

The preparation of a chlorohydroxypropyl derivative is described

and then various trial reactions are detailed.

Reaction of LH-20 with 3-chloro-oxidopropane.

LH-20 (21 g) was equilibrated for 1 h in CH_2Cl_2 (100 ml). BF_3 etherate (5 ml) was added and the suspension stirred for 15 min. 3-Chloro-oxidopropane (16 ml) in dry CH_2Cl_2 (25 ml) was added over 30 min and the reaction mixture was stirred overnight. The product was filtered, washed with CH_2Cl_2, ethanol and acetone, and dried under vacuum. The gel now weighed 34.5 g, corresponding to a chlorohydroxypropyl content of 39.1% by weight, or 2.3 replaceable chlorine atoms per sugar ring of the dextran. The average MW of a monomer in the gel was therefore 552.

Reaction of chlorohydroxypropyl LH-20 with L-ephedrine.

1.00 g of gel was suspended in dry CH_2Cl_2 (4 ml) for 1 h and then ephedrine (0.8 g) in CH_2Cl_2 (2 ml) was added and the reaction mixture left at room temperature for 6 days. The gel was filtered, washed and dried. It now weighed 1.08 g, corresponding to an uptake of 0.60 m.moles of ephedrine or 0.33 ephedrine residues per glucose ring of the dextran.

Reaction of chlorohydroxypropyl LH-20 with borneol alkoxide.

Borneol alkoxide was prepared by the addition of borneol (3 g 0.192 mole) in dry THF (5 ml) to sodium hydride (0.75 g, 0.312 mole) and allowing reaction to occur for 30 min. Chlorohydroxypropyl LH-20 (3.00 g) was dried thoroughly and suspended in dry THF (12 ml) for 30 min prior to the addition of the alkoxide. On adding the base, a greenish-yellow coloration was formed, indicating that reaction was occurring. The suspension was stirred for 2 days at room temperature, after which the gel was filtered, washed with H_2O, ethanol, acetone and benzene. The product initially had a brown colour but on continued washing, this was

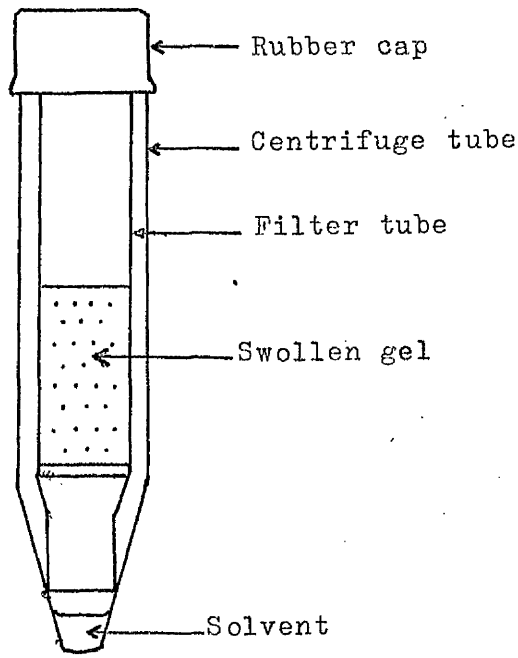


Fig.2.10 Apparatus for the determination of solvent regain values by the method of Helfferich.

partly removed. The dry gel now weighed only 2.8 g, a weight decrease of 0.2 g (6.7%).

The reaction was repeated with similar conditions and once again a decrease in weight was observed (of 6.0%).

The degree of substitution cannot be calculated as the extent to which chlorine has been displaced by hydrogen or hydroxyl is not known.

Base-catalysed reaction of LH-20 with cyclohexene oxide.

Cyclohexene oxide was used as a model compound for the reaction of more complex alicyclic oxides. LH-20 (2.0 g, 6.0 m.moles) in t-butanol (20 ml) was stirred for 5 h with potassium t-butoxide (2 g, 18 m.moles) and then excess base was filtered off. Cyclohexene oxide (5 ml) was added and the suspension was stirred for 2 days at room temperature. A green coloration developed soon after the addition of the oxide, but on continued reaction this faded to give a yellow reaction mixture. The gel was washed and dried. It now weighed only 1.84 g. Allowing for mechanical losses, this implied zero incorporation of the oxide.

2.7 Measurement of Solvent Regain Values.

Solvent regain values are expressed either as the number of grams or the volume in ml of solvent absorbed at equilibrium by 1 g of dry gel. This does not include liquid in the interstitial spaces between gel particles. The method used was adapted from that described by Pepper et al.[127] and later by Helfferich[128] for use with ion-exchange resins. Ellingboe used a similar procedure[90] for his hydroxyalkoxy derivative of LH-20.

Method: A sample of the gel under examination was weighed out into the filter-tube (Fig. 2.10) and was equilibrated with the solvent. Excess

solvent was removed by suction, until the meniscus of the supernatant had merged with the surface of the gel bed, and the container was transferred to a conical centrifuge tube which was then sealed with a rubber cap. The atmosphere inside the tube was saturated with solvent vapour by addition of a few drops of solvent to the centrifuge tube. The assembly was spun for 15 min at 150 g and then the filter tube was removed, dried quickly with filter paper, and weighed to give the weight of solvent absorbed by the gel beads. This method does not allow for solvent adhering to the surface of the particles. It was not considered necessary, for the present purposes, to compute the corrections mentioned by Helfferich.[33] Values determined for gels are quoted in Chapters 3-5.

2.8 Preparation of Columns.

It has been shown experimentally[9,129,130] that column diameters of 1-3 mm I.D. are best for high performance liquid chromatography (HPLC). Accordingly, analytical columns for the evaluation of synthetic gels were constructed from narrow bore tubing (Pyrex medium wall, 2.5 mm I.D. or P.T.F.E. capillary tubing 1.6 mm I.D.). Column lengths were approximately 100 cm. Straight columns were used in preference to coils, which have been shown to be less efficient.[35,37] Preparative-scale columns were made from wider diameter tubing (1.0-2.5 cm). All glassware was silanised before use with dichlorodimethylsilane in toluene. The application of low pressures (5 - 10 psi) to columns gave convenient flow rates of 1 - 2 ml/h. Higher pressures could not be used because of the non-rigid nature of the gels. Brief descriptions follow for the construction and packing of both gravity-flow and pressurised columns. Packing of columns was a critical procedure requiring great care, as the

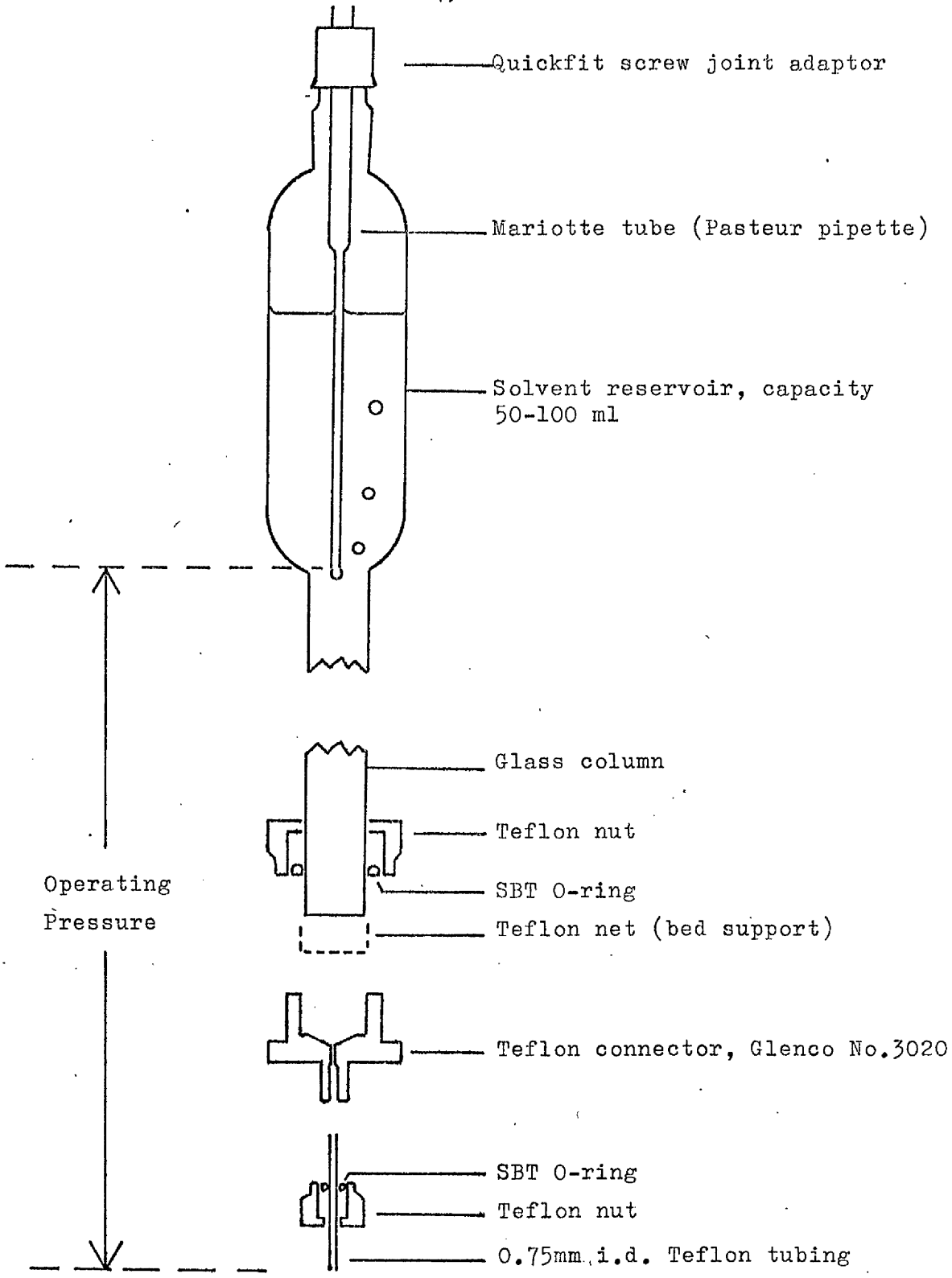


Fig.2.11 Features of the construction of gravity-flow gel columns.

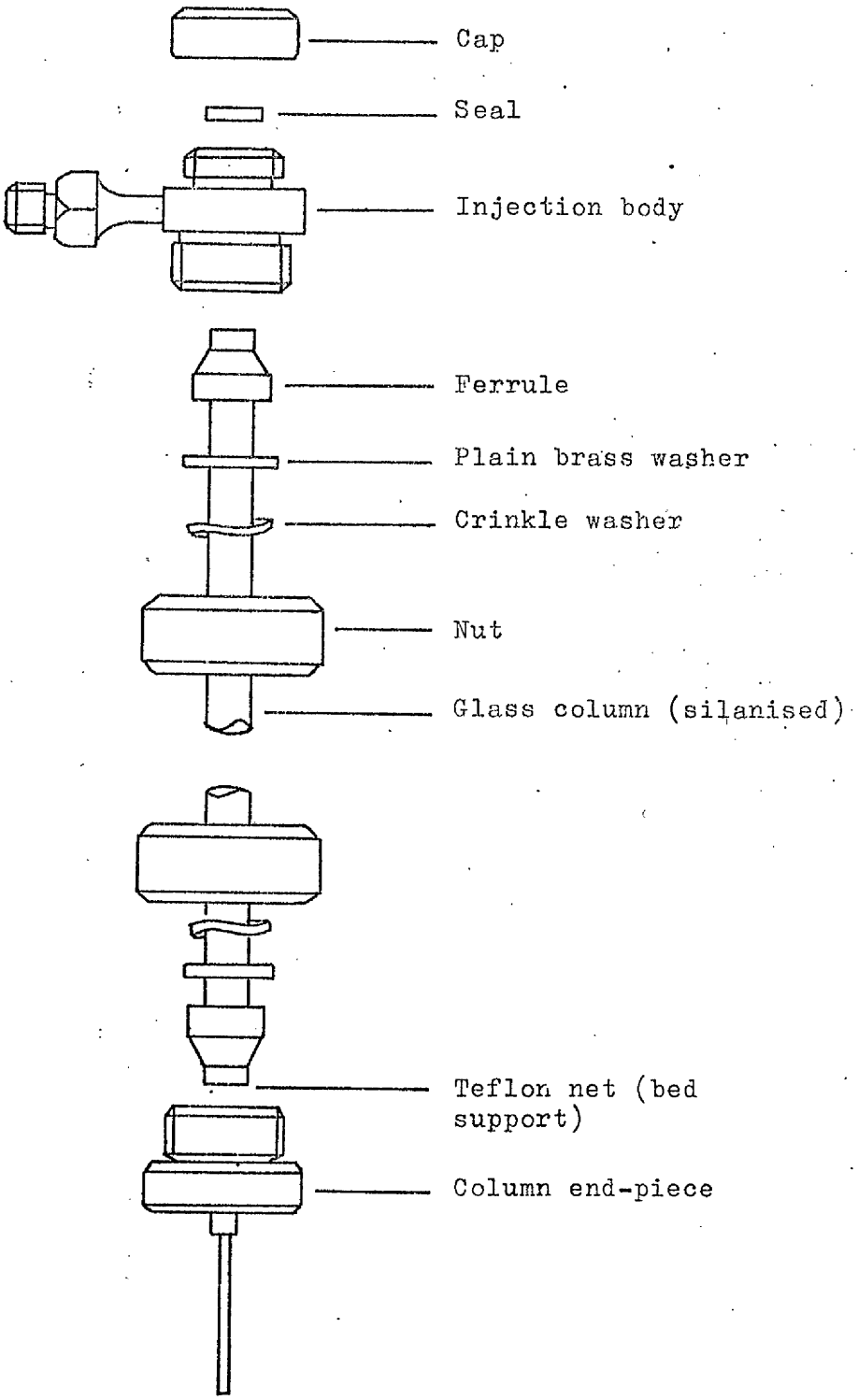


Fig.2.12 Construction of pressurised columns using Pye-Unicam Ltd. column end fittings (Injection Head Cat.No.14741, Coating Block Connector Cat.No.14743).

performance of the column was markedly affected by non-uniformity in the gel bed.

(a) Gravity-flow columns: these were constructed according to a previously described pattern,[92] utilising Glenco P.T.F.E. connectors (from Glass Engineering Co., Inc., Houston, Texas, U.S.A.) as column end-pieces. Fig. 2.11 is reproduced from ref. 38 and illustrates the column design. The Mariotte tube serves to maintain a constant operating pressure during the course of a chromatogram. Before packing, columns were calibrated in ml to facilitate measurement of bed volumes. Preparative-scale columns were packed by adding a slurry of the preswollen gel in the eluting solvent to the column and allowing the gel to sediment under gravity flow until 3 bed volumes of solvent had been collected. Analytical columns were filled beforehand with solvent and the gel slurry was added to the reservoir. This avoided the formation of air-locks in the narrow bore tubing. For both types of column, care was taken to keep the tube perpendicular. Periodic rotation of the column and gentle mechanical vibration helped in obtaining uniform sedimentation.

(b) Pressurised columns: pressure was applied to the solvent by one of the following methods: (i) from a gas cylinder attached through a regulator to the solvent reservoir bottle; or (ii) using a syringe pump (Type 355, Sage Instruments, Inc., White Plains, N.Y., U.S.A.) in conjunction with a 30-ml Hamilton syringe (No. 1030), which was connected to columns using a Teflon needle. Glass columns were fitted with Pye-Unicam injection heads and column endpieces. The washers, locking nut and ferrule were filed to accommodate the glass tubing used for the column. The ferrule on the endpiece held in place a Teflon net (Pharmacia) used as a bed support (Fig. 2.12). Capillary columns were constructed

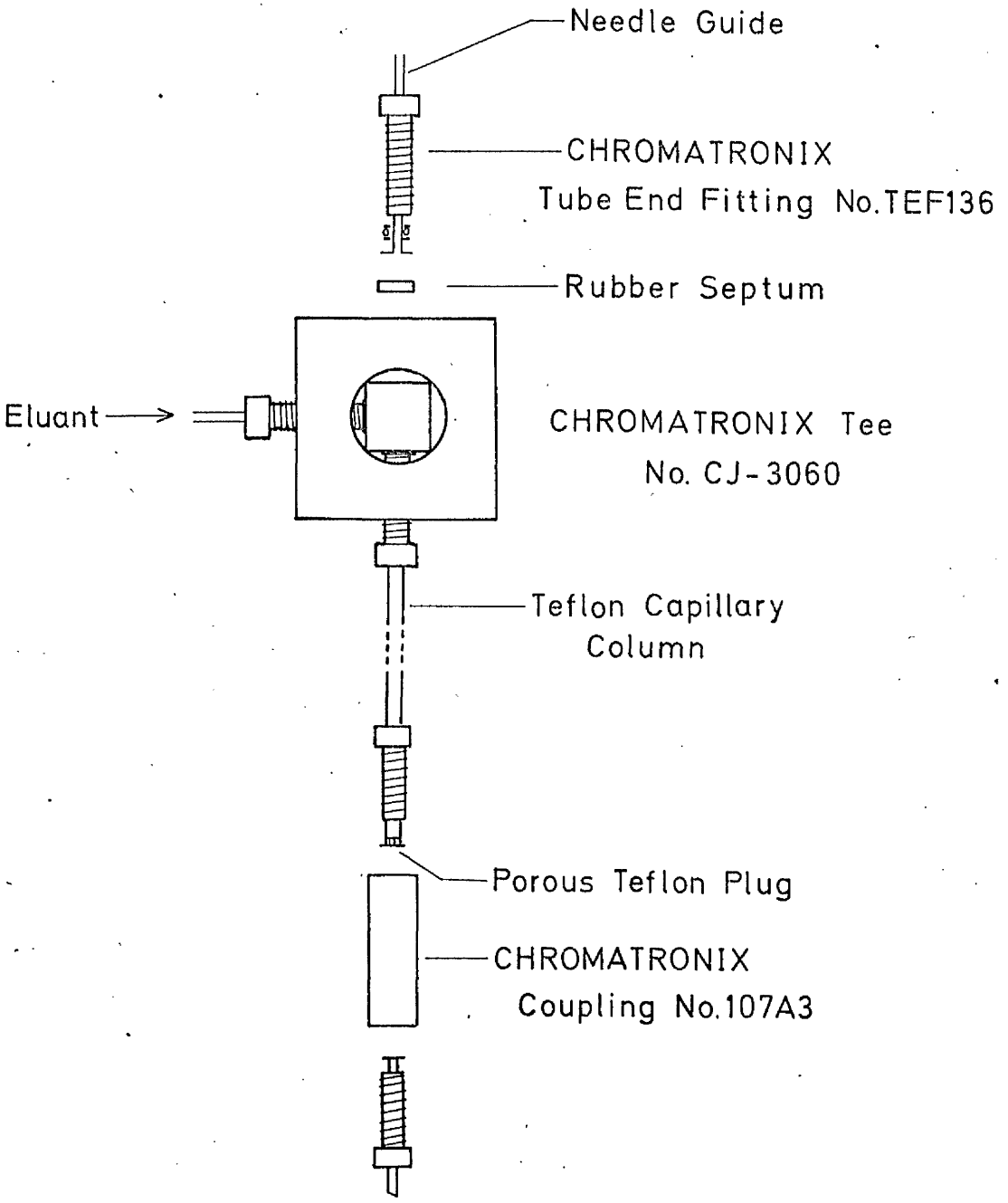
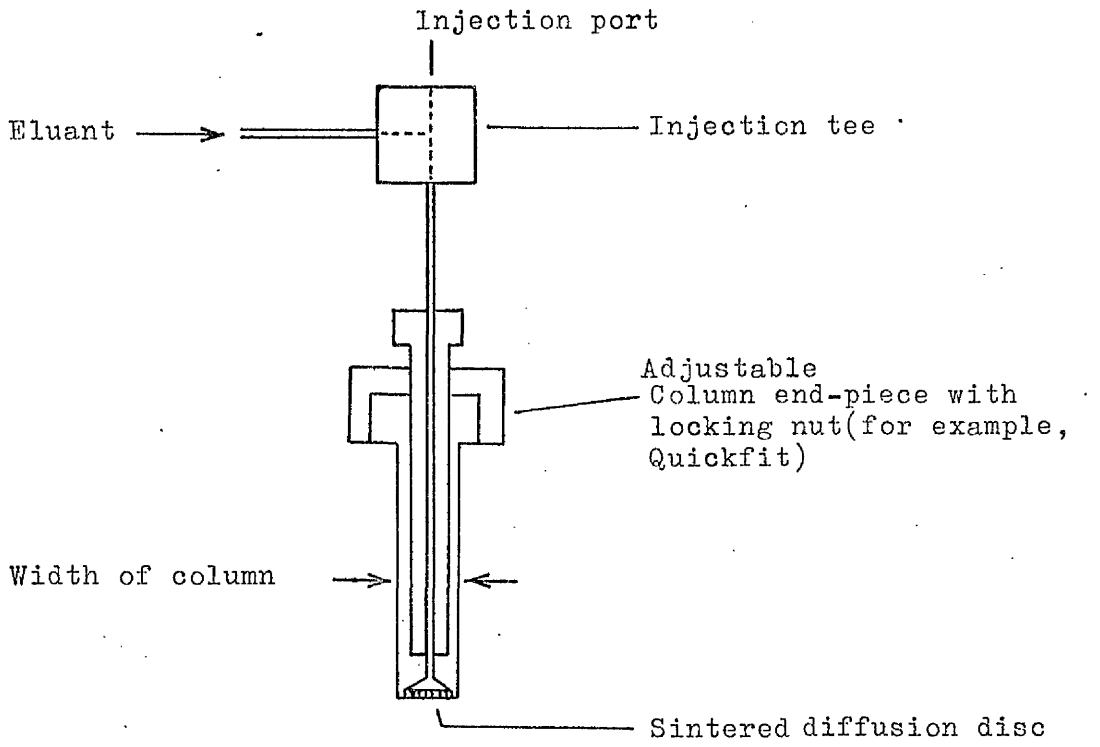


Fig.2.13 Construction of narrow-diameter gel columns using Teflon tubing and Chromatronix couplings. The flexible columns can be coiled for easier handling.

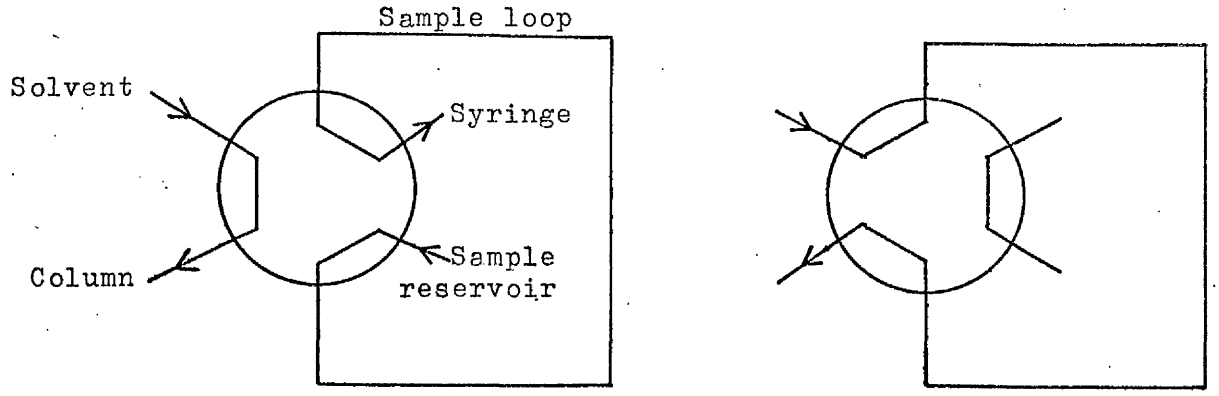
from P.T.F.E. tubing (1.6 mm I.D.) as illustrated in Fig. 2.13. Chromatronix Inc. (2743 Ninth Street, Berkeley, California 94710) valves and couplings were convenient for the construction of solvent delivery and sample injection systems.

The packing of pressurised columns required the attachment of a reservoir to the column head, through which the gel slurry was introduced to the column. As with gravity-flow columns, sedimentation of the gel was facilitated by filling the columns, initially, with solvent. The operating pressure was applied (from a nitrogen gas cylinder) to the reservoir during the packing operation. When packing was complete, the reservoir was detached and the top-piece fitted. It was convenient to maintain continuous solvent flow through columns which were liable to be needed, as some difficulty was experienced in preventing the gel bed from drying out. When this occurred, the gel was extruded from the column by reversing the solvent flow and the column re-packed once more.

Latterly, commercial columns were purchased (Quickfit) which were fitted with adjustable column end-pieces. Bed volume changes resulting from solvent switching could be accommodated with these end-pieces. In addition application of preparative-scale samples through the diffusion disc in the column top-piece resulted in narrower band widths than were obtained by conventional sample-application techniques.[92] These columns could be fitted with water-jackets for isothermal operation. However, these were not used, and chromatography was carried out in a constant-temperature room, thermostated at 20°C. The degree of swelling of the gels and the retention behaviour of samples is temperature-dependent. This will be discussed in Chapter 3.



(a)



Position I : elution of column and filling of sample loop.

Position II : addition of sample to column

(b)

Fig.2.14 Methods of sample application. For on-column injection heads, see Figs.2.13 and 2.12.

2.9 Column Techniques: Sample Application and Detection.

The development of column packing materials of high efficiency has been accompanied by advances in the techniques and equipment used for introducing and detecting samples.[5c] Poor sample application techniques can result in band spreading and dilution of the solute in the column effluent. Dilution in this manner is a problem in classical LC systems as detection is rendered more difficult. Care must be taken, therefore, to reduce dead volumes in both of the column end-pieces.

(i) Sample application.

Two methods of application are currently employed for pressurised systems: through injection ports or through sample valves. Injection ports can be of two types, on-column or swept injection ports (Fig. 2.14). In the former method, the needle of the syringe is introduced into the column through a septum and penetrates the top of the column packing. The sample is applied directly to the centre of the bed. Clogging of the syringe by very small diameter particles in the packing material can be avoided if the injection is made into a small plug of glass wool at the top of the bed. This also avoids contamination of the bed with dislodged septum material. This method can give the highest separation efficiency, especially if k' for the sample is less than unity.[5c] The sample size which can be used while maintaining high efficiency depends on the cross-sectional area of the column and the capacity of the packing. Kirkland has shown[130] that on a 1 m x 2.1 mm I.D. column packed with a controlled surface porosity support, the HETP value does not increase significantly over the range 0 - 50 µl. This method of sample application was found satisfactory for all types of analytical column described in paragraph 2.8 above.

When a swept injection port is used, the sample is deposited just before the column inlet and is carried on to the bed by the mobile phase. This type of inlet is required for columns in which the bed is held between porous discs, as for example in the Quickfit columns mentioned earlier. The efficiency can be as high as for on-column application if dead volumes are minimised and also if k' is greater than unity. With both injection systems, the flow can be interrupted during sample application. Band broadening is not a problem, as diffusion coefficients in liquids are low, and in addition, this method allows low pressure syringes to be used. It is preferred for large sample volumes or for systems with very high operating pressures ($>$ 2000 psi). Alternatively, these problems can be avoided using a sample valve (or sample loop, Fig. 2.14). These valves are commonly used for automated repetitive analysis, where high reproducibility of sample introduction is required. A design has been published for a valve which can be operated at up to 5000 psig.[132]

Gravity-flow preparative columns were loaded in the normal manner, by removing supernatant solvent prior to applying the sample.

(ii) Sample detection.

A wide range of detection systems have been used with liquid chromatographs.[5] None of these are "universal", in that they are suitable for all types of sample, and modern systems generally incorporate more than one detector in series with the column. Three groups can be identified.

(a) Bulk property detectors, which measure overall physical changes in the eluant, for example, the refractive index (RI), conductivity or dielectric constant (capacitance). Of these, the differential refractometer is the most commonly used. The detector is sensitive in the μg

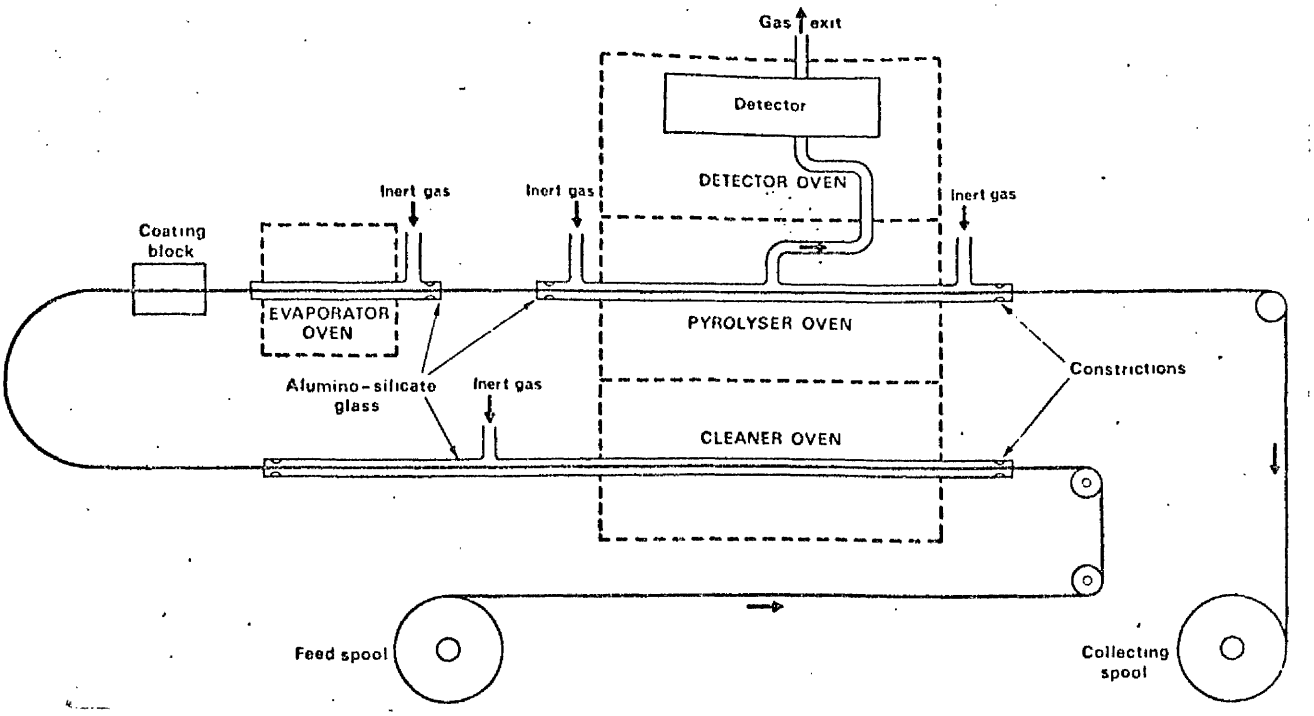


Fig.2.15(b) Schematic illustration of the Pye System 2 Liquid Chromatograph with flame ionisation detector.

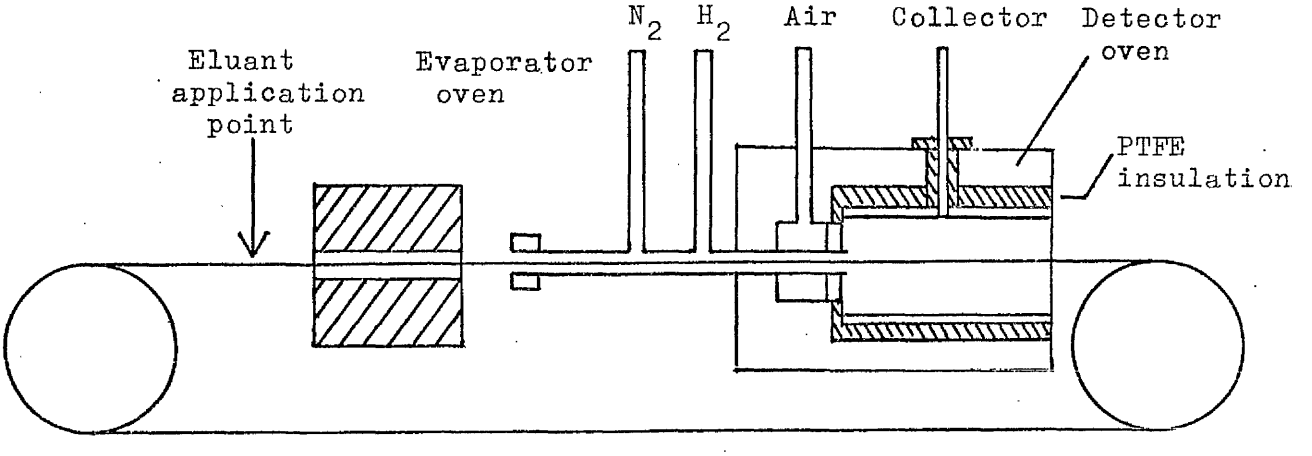


Fig.2.15(a) Construction of the Sjövall-Haahti liquid chromatographic detector (purchased from the Karolinska Institutet, Stockholm).

range but is dependent on flow rate and temperature.

(b) Solute property detectors: these depend on particular properties possessed by the sample but not the eluant, and include UV, polarographic and radioactivity detectors. The UV detector is the most widely used detector of this type. Although limited in its use to compounds containing chromophores, it has the advantage of being only very slightly affected by temperature and flow rate and of being extremely sensitive even for compounds of moderate absorptivity. In favourable cases sub-nanogram amounts of solute can be detected.

(c) Flame ionisation detectors (FID, transport detectors): the solvent is removed by evaporation on the surface of a wire transported continuously through the effluent, thereby receiving a film of the liquid, and the solute left on the wire is subsequently pyrolysed and carried to the detector in a stream of suitable gas. The detector is identical in construction to those used in gas chromatography. The sensitivity of the system is limited by the capacity of the wire and is dependent on the eluant concentration and sample volatility.

In the present work, detection of solute in column effluent was achieved almost entirely by the use of liquid chromatographs of the moving wire - FID type. One of these was based upon the design of Haahti *et al.*[133] (Fig. 2.15a) and the other was a Pye-Unicam System 2 liquid chromatograph (Fig. 2.15b). The platinum chain[133] was replaced by a wire prepared as follows. Two strands of wire (80/20 Vacrom wire, from the Factile Wire Co. Ltd., Bootle L.20 6AE, Lancs.) were twisted to give 8 turns per cm. The wire was doubled and twisted to give a 4-stranded composite wire.[92] This had a satisfactory sample capacity, a useful lifetime (1-2 weeks when in continuous use), and a low noise level after rigorous cleaning.

Sample size was commonly 25-100 μg, but 1 - 2 mg could be accommodated on columns of bed volume 10 - 20 ml without deterioration of peak shape. Elution of 25 μg of material in a peak of width 10 S.E.V. from an analytical column, bed volume 5ml, resulted in an average eluate concentration of 50 μg/ml which was easily detected on the liquid chromatographs. The background noise arising from column bleed was usually negligible. On re-equilibrating columns which had been dried out for some time, a rise in noise level was often observed. The exact cause of this is not known : impurities may have entered the column during the repacking procedure, or degradation of the gel (bacterial or chemical) in air may have occurred, giving rise to solutes in the column effluent. After a few hours, the original low level was restored. 100 ml of eluate from the hydroxycholestanol LH-20/benzene system were evaporated and the resulting oil was examined by GLC (2 m 1% OV-1 at 225°C). A solvent blank (the residue from 100 ml benzene) was compared with the effluent residue. The GLC traces of the two samples were identical.

In some cases, solutes eluted from columns were detected by analysis of fractions collected during the course of the chromatogram. Two automatic collectors were used : the BTL Chromafrac (Baird and Tatlock (London) Ltd., Freshwater Road, Chadwell Heath, Essex), and the Central Fraction Collector (Central Ignition Co., London, N.1.). In addition, very small fractions were collected manually. Solvent was evaporated from fractions with a stream of nitrogen and solid residues dissolved in an appropriate solvent for UV spectroscopy, scintillation counting or polarimetry.

In the analysis of biological extracts, some use was made of a

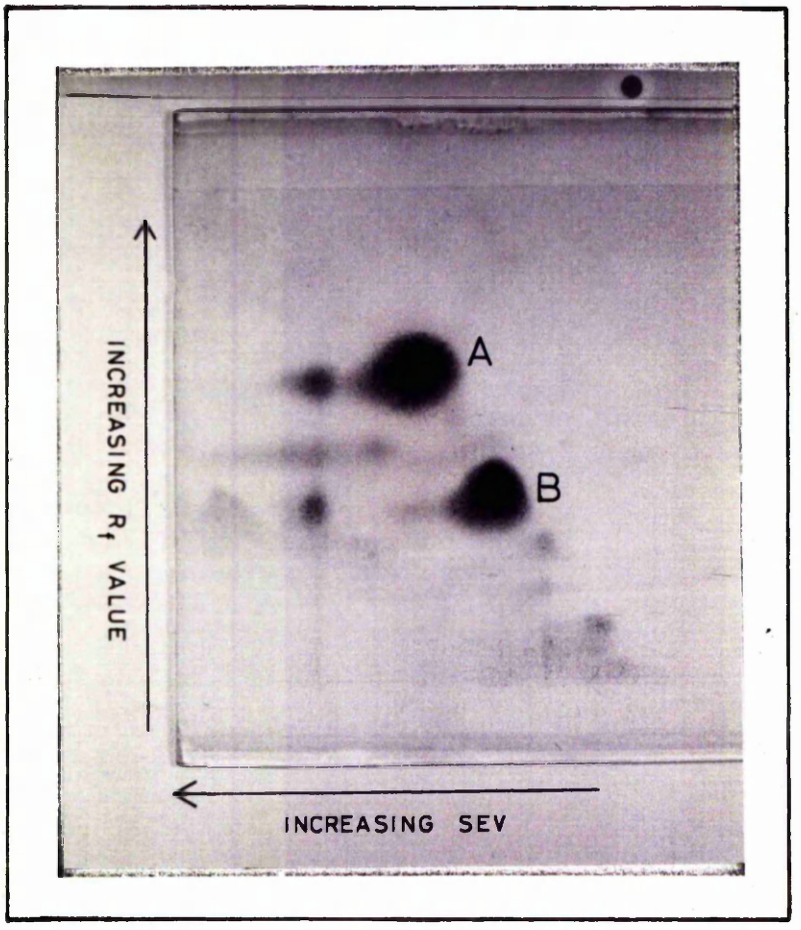


Fig.2.16 A two-dimensional chromatogram of a World Standard pyrethrum extract obtained by collecting the effluent from a gel column on a TLC plate as a streak (x'-axis). The plate was then developed (along the y-axis) in the normal manner and visualised with ceric ammonium sulphate. The system was a reversed-phase hydroxycholestanol gel eluted with methanol-heptane (9:1).

Compound A is pyrethrin I and B is pyrethrin II. The separation is similar to that obtained with reversed-phase hydroxy-5β-cholanyl LH-20 (Fig.3.7).

device for coupling column chromatography with TLC. A 20 x 20 cm TLC plate (0.25 mm thickness) was transported at 3 cm/h below a gravity-flow analytical gel column from which the effluent was deposited on the silica layer as a streak. The solvent was evaporated continuously by a stream of nitrogen directed at the point of application. The plate was then developed and visualised in the normal manner, giving a 2-dimensional chromatogram (Fig. 2.16). My thanks are extended to Mr. D.S. Stevenson for the use of this apparatus.

Chapter 3. HYDROXY-5β-CHOLANYL SEPHADEX LH-20.

3.1 Introduction.

Two approaches to the chromatographic resolution of chiral molecules were outlined in Chapter 1: (a) by chromatography of diastereoisomeric derivatives of the compounds on normal stationary phases, and (b) by direct chromatography on optically active stationary phases. By virtue of the fact that matrix substitution of Sephadex, to produce lipophilic derivatives, had successfully been carried out in a number of cases,[83,88,90,95] and also because it is more convenient to work with the heterogeneous mixtures isolated from plants without derivative formation (which might result in rearrangement reactions of labile compounds), it was decided to adopt the second method (b) above.

Certain requirements are imposed on reagents for gel modification reactions.

(i) It must be possible to introduce substituents without adversely affecting the gel structure. If very vigorous alkaline or acidic conditions are used in the substitution reaction, the danger arises of hydrolysis of the polysaccharide matrix, with consequent deterioration in the physical properties of the gel bead. Goodson and Di Stefano[134] reported the hydrolysis of G-15 in boiling HCl (6N), although, if the extent of reaction is carefully controlled, beneficial results are claimed, in terms of column efficiency, for the partially hydrolysed structure. However, a loss of homogeneity with respect to the degree of cross-linking might arise in unfavourable reaction conditions.

(ii) The substituents must be stable with respect to physical, chemical and stereochemical properties. They must be introduced with-

out loss of chirality, and, once bound to the gel matrix, they must not readily undergo subsequent chemical reaction which would result in their racemisation or decomposition. Reactions of these types might occur with solvents or solutes passing through the column. This problem has arisen with siliceous esters, which are hydrolysed by solvent systems containing alcohols[135] - an effect that imposes limits on the use of these bonded stationary phases. Ideally a stable chiral centre containing no labile functional groups is required. This is in accord with the view proposed in Chapter 1, that separations of phytosterols diastereoisomeric in the side chain were unlikely to be achieved on polar optically active phases containing multifunctional substituents.

(iii) The material used for preparing the gel should be readily available or, at least potentially so. If the initial study of a gel showed it to be of potentially wide applicability, it would be desirable that further quantities could be prepared.

These conditions are satisfied, in terms of matrix stability and column performance by the BF_3-catalysed reaction of an olefin oxide with LH-20,[89-93,95] the gel substrate on which substitutions have sometimes been carried out in preference to Sephadex G. Substitution of the free hydroxyl groups in G-25 renders it lipophilic-hydrophilic and therefore more suitable for reaction in organic solvents with lipophilic reagents than the original hydrophilic dextran. The types of linkage which have been used to attach substituents include esters,[136] amides,[95] amines,[95] ethers,[83,88,90,95] and silyl ethers.[94] The ether linkage is to be preferred for permanent column materials. In the preparation of methylated Sephadex, Nyström used G-25 in aqueous NaOH

$$\text{Dex}-\text{OH} \xrightarrow{(CH_3CO)_2O,\ \text{Benzene}} \text{Dex}-\text{O}-\text{CO}.CH_3 \qquad (103)$$

$$\left.\begin{matrix}\text{Dex}-\text{OH} \\ \text{Dex}-\text{OH}\end{matrix}\right\} \xrightarrow[\text{NaH/DMSO (105)}]{\text{Aqueous NaOH (104)}} \text{Dex}-\text{O}^- \xrightarrow{Me_2SO_4} \text{Dex}-\text{OMe}$$

$$\text{Dex}-\text{OH} \xrightarrow{(CH_3)_3SiCl,\ \text{Pyr.},\ \text{Toluene}} \text{Dex}-\text{O}-\text{Si}(CH_3)_3 \qquad (94)$$

$$(\text{LH-20})-\text{OH} \xrightarrow{ClCH_2-\underset{\diagup\ \diagdown}{CH}-CH_2,\ BF_3} (\text{LH-20})-\text{O}-\text{CH}(CH_2OH)CH_2Cl \qquad (95)$$

$$(\text{LH-20})-\text{O}-\text{CH}(CH_2OH)CH_2Cl \xrightarrow{C_2H_5NH_2} (\text{LH-20})-\text{O}-\text{CH}(CH_2OH)CH_2-\text{NH}.C_2H_5$$

$$(\text{LH-20})-\text{O}-\text{CH}(CH_2OH)CH_2Cl \xrightarrow{NH_3} (\text{LH-20})-\text{O}-\text{CH}(CH_2OH)CH_2NH_2$$

$$(\text{LH-20})-\text{O}-\text{CH}(CH_2OH)CH_2NH_2 \xrightarrow{R-CO_2H} (\text{LH-20})-\text{O}-\text{CH}(CH_2OH)CH_2NHCOR$$

Dex –OH represents the dextran matrix of Sephadex G. In the last equation, $R-CO_2H$ is lithocholic acid :-

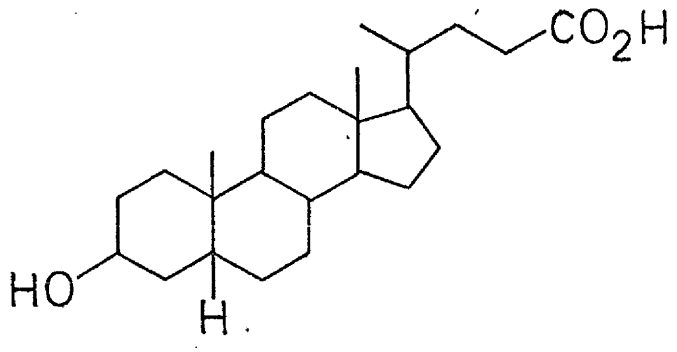


Fig.3.1 Some examples of reactions for the preparation of lipophilic Sephadex gels.

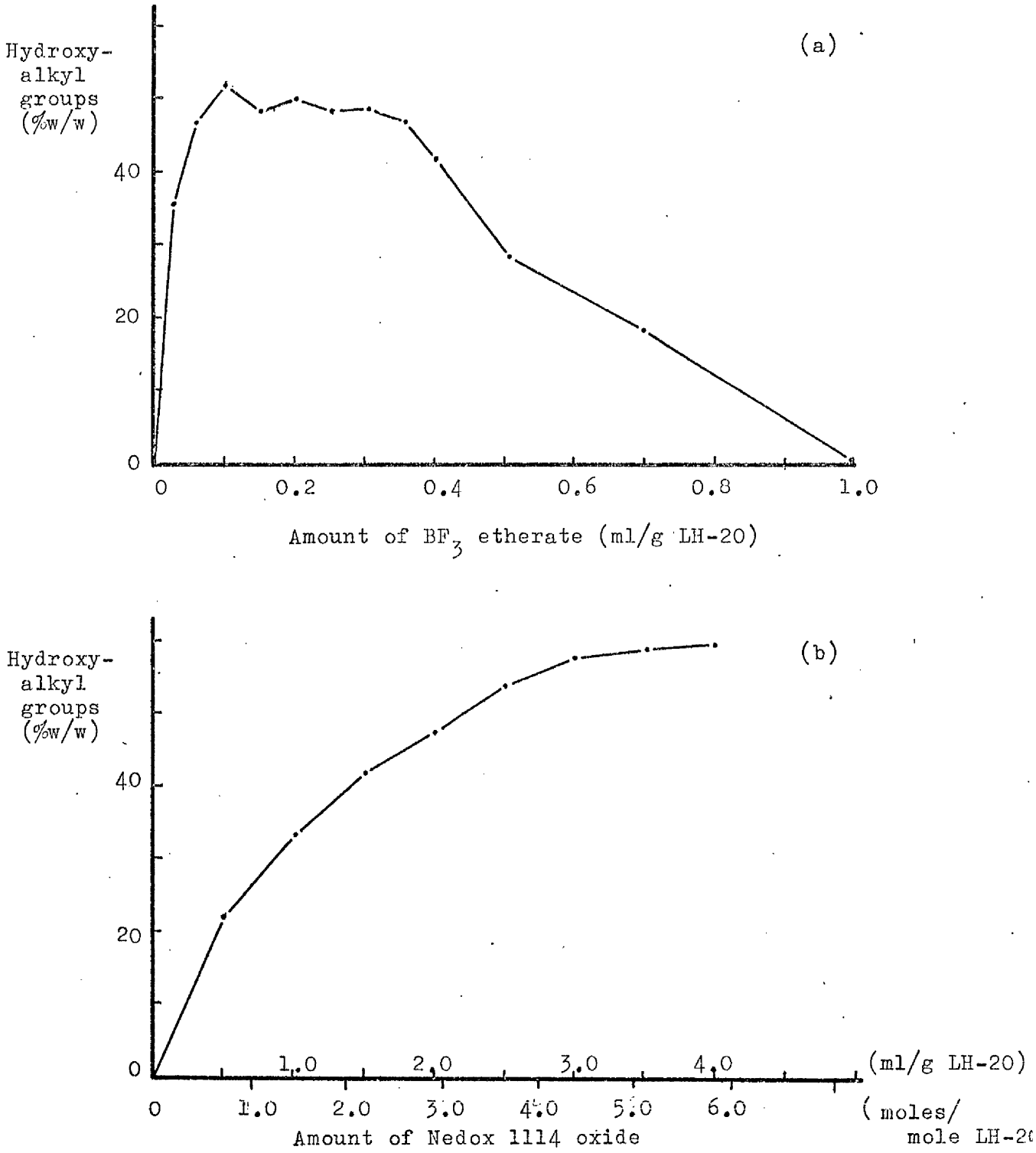


Fig.3.2 Dependence of the degree of substitution of hydroxyalkyl LH-20 on (a) the amount of BF_3 etherate, (b) the amount of oxide used. Graph (a) was determined using 2.5 ml Nedox/g LH-20 (3.57 moles/mole), and (b) was determined using 0.25 ml BF_3/g LH-20. Both graphs are reproduced from ref.90.

with dimethyl sulphate[137] as the methylating agent, or else reacted G-25 in dimethyl sulphoxide with the dimethylsulphinyl carbanion and dimethyl sulphate (Fig. 3.1).[138]

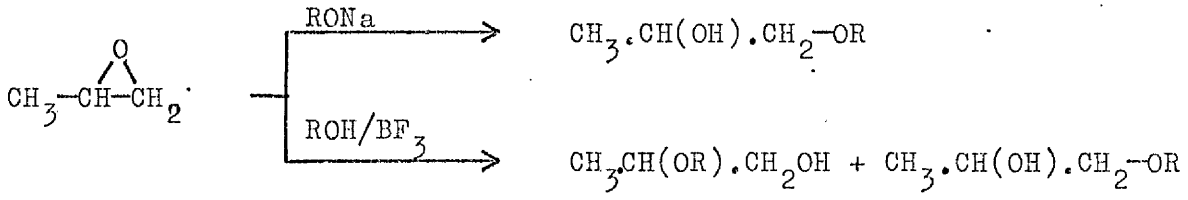
The chiral oxide chosen for the preparation of a gel was 23,24-oxido-5β-cholane. This is a terminal olefin oxide (and therefore similar in this respect to Nedox 1114 oxide) which could be prepared from naturally occurring and readily-available bile acids. The functional groups in the steroid nucleus were removed to leave a lipophilic moiety which had considerable chemical stability. The oxide was prepared from 5β-chol-23-ene by reaction with peracid. In the absence of any pronounced steric hindrance in the vicinity of the olefin above or below the plane of the steroid nucleus, it is presumed that a mixture of the α- and β-oxides was formed. No net contribution to the chirality of the substituted gel could therefore be expected from the potentially chiral centre at C-23.

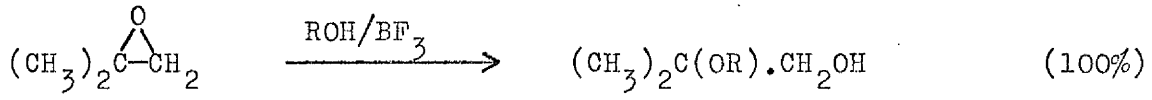
Optimisation of the experimental conditions was carried out using a mixture of Nedox 1114 oxides as model compounds. Stoichiometric amounts of reagent were used, in contrast to previous work by Nyström, who used excess oxide. Nyström examined the degree of substitution versus the amount of oxide and acid catalyst present (Fig. 3.2). From the graphs reproduced in Fig. 3.2, it can be seen that the percentage substitution of the gel increases with the amount of oxide used, reaching a maximum of 60% by weight of substituent, which corresponds to 2.64 hydroxyalkyl residues per monomer unit of the LH-20 polymer (i.e. 88% substitution of the free hydroxyl groups of LH-20). The efficiency of incorporation at this degree of substitution is only 46%. When stoichiometric amounts of oxide are used (approximately

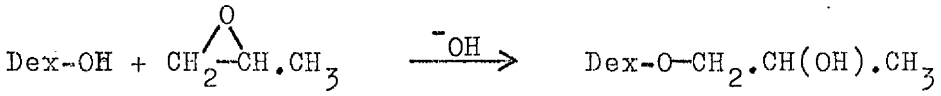
1 mole/mole of gel), the efficiency rises to 60-70%, which agrees with the findings described in Chapter 2. There is an optimum amount of BF_3 catalyst for this reaction, between 0.1 and 0.4 ml/g of LH-20. Beyond this figure, Nyström's graph describes a falling curve. He does not suggest any explanation, but from the results of the present work, it appeared that the gel had undergone severe degradation in the presence of high amounts of BF_3 etherate, a situation recalling the criteria for suitable substitution methods discussed above (3.1(i)).

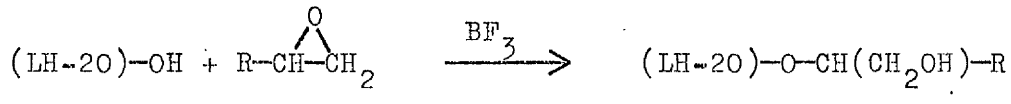
The substitution reaction with 23,24-oxido-5β-cholane was successful and an efficient incorporation of the oxide was obtained.

The exact structure of the gel following substitution is not known, and it is likely to prove very difficult to carry out a detailed analysis without degrading the polymer, for example, to single monomer units. Some work has been done on the NMR spectra of polysaccharides.[139] However, high viscosity (and the rigidity of a cross-linked polymer) results in band broadening and the structure of the substituent-gel linkage is unlikely to be given by this method. Tanaka and Konishi have published[94] an infrared spectrum of a trimethylsilyl derivative of G-100. While this shows the presence in the gel matrix of Si-Me groups, the bands once again are rather broad. However, the mode of reaction of epoxides has been well catalogued,[140-143] and the direction of epoxide opening in acid or base can be inferred from known examples. Thus when propylene oxide is reacted with sodium alkoxides, as in the preparation of LH-20, nucleophilic attack occurs only at the terminal epoxide carbon atom. When acid catalysts (BF_3 etherate, H_2SO_4) or heat are used, a mixture of primary and secondary alcohols is formed.[144] (Fig. 3.3.1). More highly substituted oxides tend to give predomin-

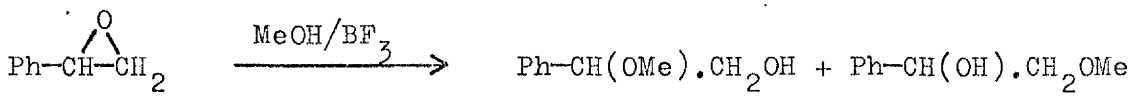
(1) 

(2) 

(3) 

(4) 

R—CH—CH$_2$ = 23,24-oxido-5β-cholane

(5) 

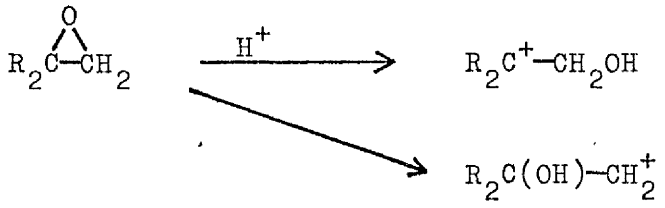
(6) 

Fig.3.3 Acid and base-catalysed nucleophilic attack on epoxides.

antly the primary alcohol. For example, isobutylene oxide gives only 2-methoxy-2-methyl-1-propanol (Fig. 3.3.2) when reacted with BF_3 in methanol. It is reasonable to assume that LH-20 contains secondary hydroxyl groups (Fig. 3.3.3) and that BF_3-catalysed reaction with 23, 24-oxido-5β-cholane results mainly in attack at C-23, to give a primary hydroxyl group (Fig. 3.3.4). The direction of epoxide opening is apparently controlled by the relative stabilities of the two carbonium ions formed transiently in acid-catalysed reactions (Fig. 3.3.6). A double bond or aromatic ring in conjugation with the epoxide exerts a strong directional influence by stabilising the ion through delocalisation of the charge. The amount of secondary alcohol formed during the substitution reaction will also depend on the effects of steric hindrance on attack at C-23 or C-24.

The reaction supernatant was analysed by GC after substitution had occurred. The residual steroidal material was shown to comprise over 97% (estimated by triangulation of the peaks) of a by-product of the synthesis of the olefin, 24-butoxy-5β-cholane. This had not been removed from the olefin, as it was considered that it would not interfere with subsequent reactions. Epoxide rearrangement products appear to be absent in this reaction (cf. Chapter 4).

3.2 General Properties of the Gel.

Hydroxy-5β-cholanyl LH-20 is a chemically stable gel. Examination of a sample by optical microscopy showed that the spherical form of the beads had been preserved through the modification reactions. Column bleed from analytical scale columns, in terms of background noise on the liquid chromatograph, has been found to be negligible with

TABLE 3.1 Solvent regain values for hydroxy-5β-cholanyl substituted LH-20* and Nedox 1114 substituted LH-20**

Solvent	Solvent Regain Value†	
	Cholanyl LH-20	Nedox 1114 LH-20
Heptane	0.84	0.5
Cyclohexane	1.25	
Carbon tetrachloride	2.77	
Toluene	1.88	
Benzene	2.41	1.4
Tetrahydrofuran	2.19	
Chloroform	3.67	
Methylene chloride	2.75	
Acetone	1.04	
Methanol	1.16	0.7

* Degree of substitution 40.3% by weight.

** Degree of substitution 41.3% by weight.

† Grams of solvent taken up by 1 g dry gel.

both polar and non-polar solvent systems. This agrees with observations made by Ellingboe et al. on the properties of Nedox 1114 LH-20, especially after rigorous washing of the gel.[90] Elution data were reproducible over a period of several months.

Solvent regain values (SRV) are quoted in Table 3.1. Maximum solvent uptake is obtained with halogenated and with aromatic solvents, but swelling does occur with more polar solvents. Sjövall has attributed the degree of swelling to four main factors:-[83] (a) the degree of cross-linking; (b) the degree of substitution; (c) the type of substituent; (d) the nature of the solvent. Following from the discussion in Chapter 1, of the nature of gel polymers, it is expected that (a) is the primary factor. As the weight of lipophilic substituent increases, the solvent regain in non-polar solvents increases while, in polar solvents, it decreases. Thus hydroxyalkylated LH-20 is hydrophobic even at low degrees of substitution. Studies of methylated Sephadex,[83] LH-20,[83,88] Nedox 1114 LH-20[90] and trimethylsilyl Sephadex have shown that these trends are general. The numerical values of the SRV for cholanyl LH-20 are higher than those for hydroxyalkyl LH-20 of a similar degree of substitution[90] (Table 3.1, column 2). Procedural differences during the determination, as for example in the speed and time of centrifugation, may account for this. The relative values for different solvents with the same gel are therefore more significant.

Little information is at present available on the effects of temperature on lipophilic gels and on the SRV. Lampert and Determann have looked at the effect of temperature on the porosity of LH-20 (measured by the degree of swelling)[145] and on the elution times of materials from columns of LH-20.[146] The solvent uptake increases with

TABLE 3.2 % Recovery of materials of different polarity from the straight-phase system.

Compound	load	dpm added	SEV Range collected	dpm recovered	% recovery
Cholesteryl palmitate	10 µg	33636	50-60	33126	99
Cholesterol	10 µg	4067	132-148	4096	101
Palmitic acid	10 µg	43938	200-250	43169	98

temperature for organic solvents and decreases for water or electrolyte solutions. The elution volume of organic acids in an electrolyte system increases with temperature, whereas, in an organic solvent, it decreases. This effect has been correlated with the mechanism underlying separation and will be discussed later (Chapter 5). Hydroxyalkyl LH-20 decomposes at temperatures over 80°C.[90] Also, Nyström found that elution volumes tended to decrease on hydroxyalkyl LH-20 in polar solvent systems as the temperature increased, and Ellingboe reported[90] that the theoretical plate height in polar solvents decreased on increasing the temperature. From the last result, it can be inferred that an increase in the ratio of Vs/Vm has been obtained (which leads to a more efficient column) and therefore that the porosity of the gel has increased. Hydroxy-5β-cholanyl LH-20 would be expected to follow these trends. An increase in SRV and a decrease in the elution volume are predicted for higher temperatures in the reversed-phase system. As with all partition systems, chromatography at constant temperature is necessary for reproducibility of elution data.

The gel was examined in two solvent systems: benzene and methanol-heptane (9:1), which gave straight- and reversed-phase systems, respectively. These solvent systems were chosen to facilitate comparison of the gel as a stationary phase with hydroxyalkyl LH-20.[90,92,100] One direct consequence of the lower solvent uptake with polar solvents - which also applies to other lipophilic gels - is that the size of the gel beads is smaller in reversed-phase systems than in straight-phase ones. Column flow-rates therefore tend to decrease, whereas resolution is enhanced.

Recovery of material from the straight-phase system was assessed by use of radioactively-labelled compounds of diverse polarities (Table 3.2). Virtually quantitative recovery was obtained in each case at the 10 μg

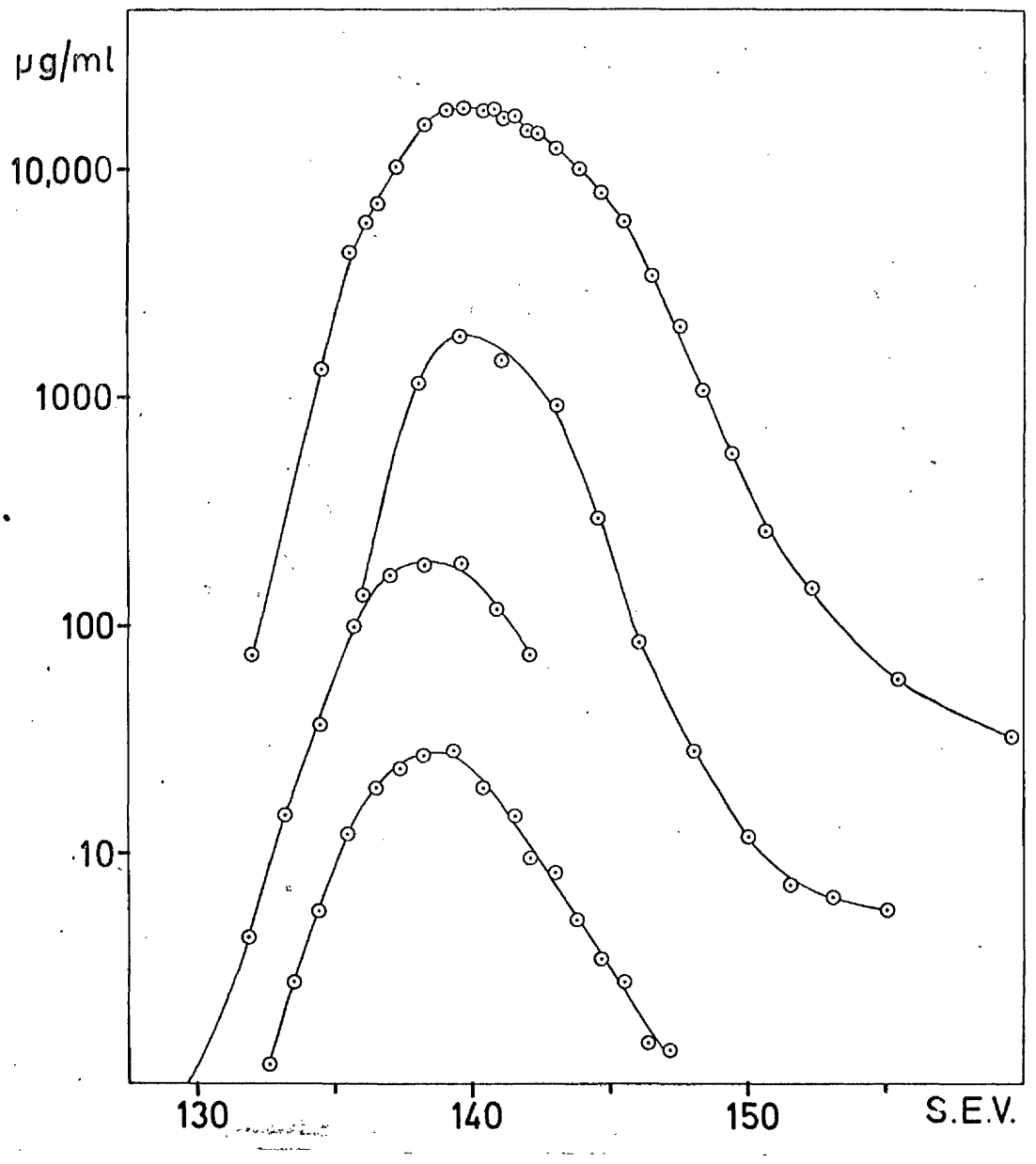


Fig.3.4 The effect of increasing the load applied to a gel column (500 X 4 mm i.d.) on the elution profile. The solute, cholesterol, contained 4-^{14}C-labelled material. Concentrations were measured through the activity of collected fractions. The total loads applied were 10µg, 100µg, 1.000µg, and 10,000µg.

level. Nyström observed 82% recovery, on average, of 15-50 ng of ^{3}H-prostaglandin E_2,[93] and Holmdahl recovered 90-97% of neutral steroids in the range 0.1-5 ng from columns of hydroxyalkyl LH-20.[147] Irreversible adsorption of solutes on gels is generally uncommon but some examples can be found in the literature.[148] Thus Stevenson reports the loss of small amounts of protein on freshly packed columns of Sephadex,[149] and George has observed the adsorption of heavy metal ions.[150]

The elution profile of cholesterol was examined as a function of load (Fig. 3.4). Fractions were collected during the elution of the solute, which contained ^{14}C-labelled material and concentrations were measured through the specific activity. The total loads applied were 10 μg, 100 μg, 1000 μg and 10,000 μg, of specific activity 0.1 μCi/mg. Increasing the load broadens the band on the column, but the almost symmetrical peak shape is maintained very well through the 1000-fold increase in the sample size. Even at high loads, "tailing" is not a serious problem. Generally, non-polar and moderately polar compounds (for example, those with one hydroxyl group) had satisfactory chromatographic profiles in the straight-phase gel system. Serious tailing was observed with more polar compounds and such materials were markedly retarded on the gel. These problems could be circumvented either by derivatisation of polar groups or by the adoption of a reversed-phase system. Brooks and Keates found symmetrical peaks with hydroxyalkyl LH-20 over a 20-fold load increase in the straight-phase system,[92] and also observed quantitative recovery of 200 μg 24-^{14}C-cholic acid from a gel filtration system.

TABLE 3.3 SEV Data for the straight-phase system.

Compound	Mol. Wt.	Standard Elution Volume	
		Cholanyl Gel	Nedox Gel
Cholesterol	386	139	110
Cholestanol	388	132	
Coprostanol	388	118	
Epicholestanol	388	132	
Epicoprostanol	388	118	
Dihydrobrassicasterol	400	133	
Campesterol	400	133	109
β-Sitosterol	414	133	107
Stigmasterol	412	129	105
Poriferasterol	412	129	
Lanosterol	426		82
5β-Cholan-24-ol	346	147	
17α-Ethynylestr-4-en-17β-ol	284	130	
Norethisterone	298	133	
19-Nortestosterone	274	150	
Estradiol 3-methyl ether	286	127	
Testosterone	288	140	
Androsterone	290	123	
Epiandrosterone	290	126	
Etiocholanolone	290	133	
3β-Hydroxy-5β-androstan-17-one	290	122	
Progesterone	314	78	61.3

Compound	Mol. Wt.	Standard Elution Volume	
		Cholanyl Gel	Nedox Gel
Cholestanone	386	70	
Cholest-4-ene-3-one	384	76	
Cholest-5-ene-3-one	384	76	
Cholest-4-ene-3,6-dione	398		586
5α-Cholestane	372	65	63
n-Octacosane	394	62	
Cholesteryl acetate	428		56
Cholesteryl butyrate	456	62	55
Cholesteryl palmitate	624	55	49
Cholesteryl benzoate	490	68	54
Cholestanyl acetate	430	65	
Epicholestanyl acetate	430	65	
Coprostanyl acetate	430	65	
Epicoprostanyl acetate	430	65	
Dihydrobrassicasteryl acetate	442	62	
Campesteryl acetate	442	62	
Stigmasteryl acetate	454	67	55
Poriferasteryl acetate	454	67	
Lanosteryl acetate	470	69	
Lanostenyl acetate	468	69	
Methyl deoxycholate	406	210	

Compound	Mol. Wt.	Standard Elution Volume	
		Cholanyl Gel	Nedox Gel
n-Decan-1-ol	158	137	
n-Dodecan-1-ol	186	129	
n-Tetradecan-1-ol	214	124	113
n-Hexadecan-1-ol	242		108
n-Octadecan-1-ol	270		103
n-Eicosan-1-ol	298	107	99
n-Docosan-1-ol	326	104	95
n-Tetracosan-1-ol	354	96	91
n-Hexacosan-1-ol	382	93	88
Trimyristin	812	54	
Tripalmitin	848	44	43.2
Tristearin	884	36	42.2
D-Usnic acid	344	79	
L-Usnic acid	344	79	
D-Ephedrine	165	208	106
L-Ephedrine	165	208	
L-Norephedrine	151		189
D-Fenchone oxime	169	87	
L-Fenchone oxime	169	87	
D-Fenchone 2,4-DNPH	334	69	
D-Ephedrine N,O-diacetate	265	70	
L-Ephedrine N,O-diacetate	265	70	

TABLE 3.4 SEV Data for the reversed-phase system.

Compound	Mol. Wt.	Standard Elution Volume	
		Cholanyl Gel	Nedox Gel
Cholesterol	386	309	250
Cholestanol	388	320	262
Coprostanol	388	391	
Epicholestanol	388	320	
Epicoprostanol	388	391	
Dihydrobrassicasterol	400	314	
Campesterol	400	314	270
β-Sitosterol	414	324	294
Stigmasterol	412	317	256
Poriferasterol	412	317	
Lanosterol	426	366	242
5β-Cholan-24-ol	346	109	
5α-Androstan-3α-ol	276	159	
Pregn-5-ene-3β-ol	302	209	
17α-Ethynylestr-4-en-17β-ol	284	143	
Norethisterone	298	80	
Mestranol	310	149	
Estr-4-ene-17β-ol	260	156	
19-Nortestosterone	274	80	
Estradiol 3-methyl ether	286	156	
Testosterone	288	79	
Androsterone	290	84	

Compound	Mol. Wt.	Standard Elution Volume	
		Cholanyl Gel	Nedox Gel
Epiandrosterone	290	84	
Etiocholanolone	290	84	
3β-Hydroxy-5β-androstan-17-one	290	84	
Progesterone	314		54
5α-Androstan-3-one	274	171	
Cholestanone	386	309	354
Cholest-4-ene-3-one	384	248	240
Cholest-5-ene-3-one	384	311	353
Cholesta-1,4-diene-3-one	382	199	
Cholesta-4,6-diene-3-one	382	199	
Cholest-4-ene-3,6-dione	398	149	136
5α-Cholestane	372	833	
Cholesteryl acetate	428		541
Cholesteryl palmitate	624	480	
Cholestanyl acetate	430	486	
Epicholestanyl acetate	430	486	
Coprostanyl acetate	430	434	
Epicoprostanyl acetate	430	434	
Dihydrobrassicasteryl acetate	442	440	
Campesteryl acetate	442	440	
Stigmasteryl acetate	454	442	

Compound	Mol. Wt.	Standard Elution Volume	
		Cholanyl Gel	Nedox Gel
Poriferasteryl acetate	454	442	
5β-Cholan-24-ol	346	109	
5β-Cholane-3,24-diol	362	77	
5β-Cholane-2,12,24-triol	378	63	
5β-Cholane-3,7,12,24-tetrol	394	49	
n-Octan-1-ol	130	104	
n-Octan-2-ol	130	104	
n-Decan-1-ol	158	145	
n-Dodecan-1-ol	186	153	
n-Tetradecan-1-ol	214	167	
n-Hexadecan-1-ol	242	182	
n-Octadecan-1-ol	270	201	
n-Eicosan-1-ol	298	224	
n-Docosan-1-ol	326	255	
n-Tetracosan-1-ol	354	293	
n-Hexacosan-1-ol	382	342	
Trimyristin	812	940	
Tripalmitin	848	1180	
Tristearin	884	1410	
D-Phenylalanine	165	53	

Compound	Mol. Wt.	Standard Elution Volume	
		Cholanyl Gel	Nedox Gel
L-Phenylalanine	165	53	
D-Phenylalanine methyl ester	179	81	
L-Phenylalanine methyl ester	179	81	
D-Ephedrine	165	63	
L-Ephedrine	165	63	
D-Mandelic acid	152	57	
L-Mandelic acid	152	57	
D-Ephedrine N,O-diacetate	265	58	
L-Ephedrine N,O-diacetate	265	58	
Pyrethrin I	328	89	
Pyrethrin II	372	80	

3.3 Chromatography on hydroxy-5β-cholanyl LH-20.

Performance of the gel proved satisfactory for a wide range of sample types. Tables 3.3 and 3.4 contain elution data for a model set of compounds in the form of Standard Elution Volumes (SEV). This term is defined[91,92] by equation 3.1:

$$\text{Standard Elution Volume} = \frac{\text{Measured Elution Volume}}{\text{Column Bed Volume}} \times 100 \quad (3.1)$$

It is a dimensionless quantity, and is independent of the column dimensions. Data from different sources can therefore be correlated, provided the gel materials used to prepare the columns are similar. It is equivalent to the expression, percentage of total column volume, introduced by Sjövall and co-workers.[83,90] For comparison purposes, SEV values recorded for hydroxyalkyl LH-20 have been included.[92,100,151] It is at once apparent that the order of elution is similar for both types of gel.

In the straight-phase system, hydroxylic, amino, and especially acidic compounds are retarded on the cholanyl-substituted gel. Carbonyl groups do not have a noticeable effect on elution volumes in this system: ketones and esters generally have similar elution volumes to the hydrocarbon analogues: for example, cholesterol, cholesteryl butyrate, 5α-cholestan-3-one, and 5α-cholestane have SEV values 139, 62, 71 and 65 respectively. Selective retention of aromatic compounds has been observed in Sephadex G systems[152-156] and on LH-20 in alcohol-based solvent systems[152,157,158] (but not in chloroform-bases systems[159]). Makowetz et al., for example, separated and determined thyroid hormones on the basis of this interaction,[160] and several other similar applications have been published (see ref. 148 and references cited therein).

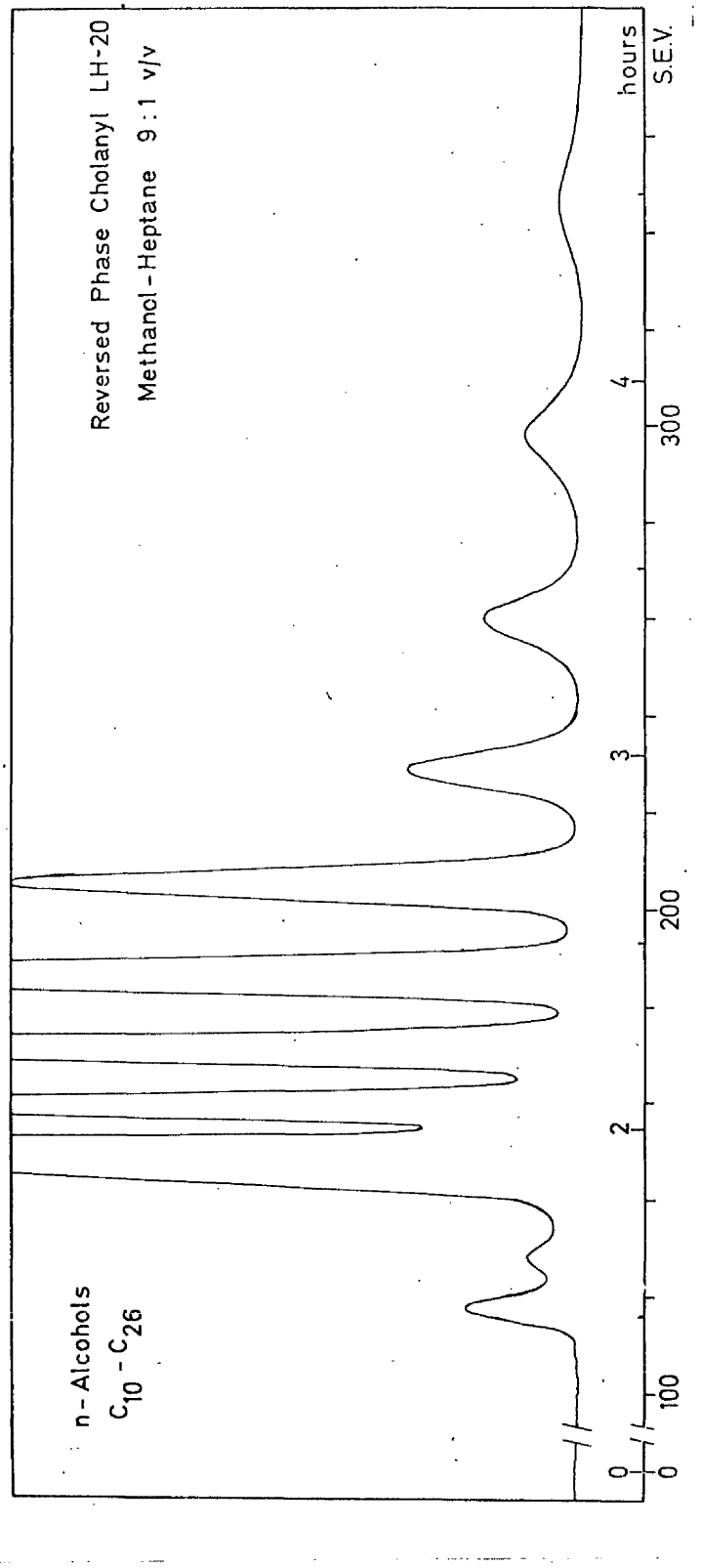


Fig.3.5 Chromatography of n-alcohols of even carbon number, C_{10}—C_{26}, on a reversed-phase cholanyl LH-20 column (700 X 1.6 mm i.d.) eluted with methanol-heptane (9:1) and detected with a liquid chromatograph by moving wire-FID. The alcohols are eluted in increasing order of molecular weight. The small peaks in the trace below S.E.V. 140 arise from impurities.

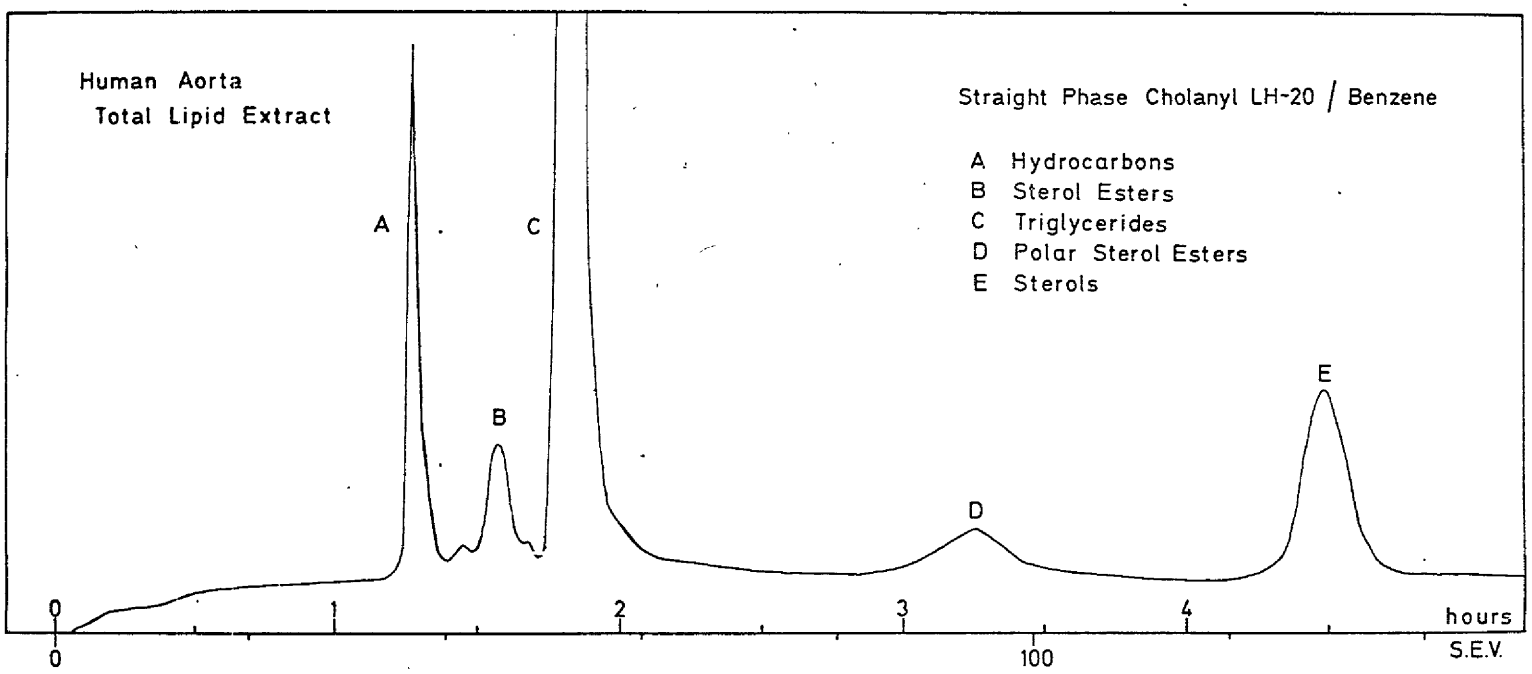
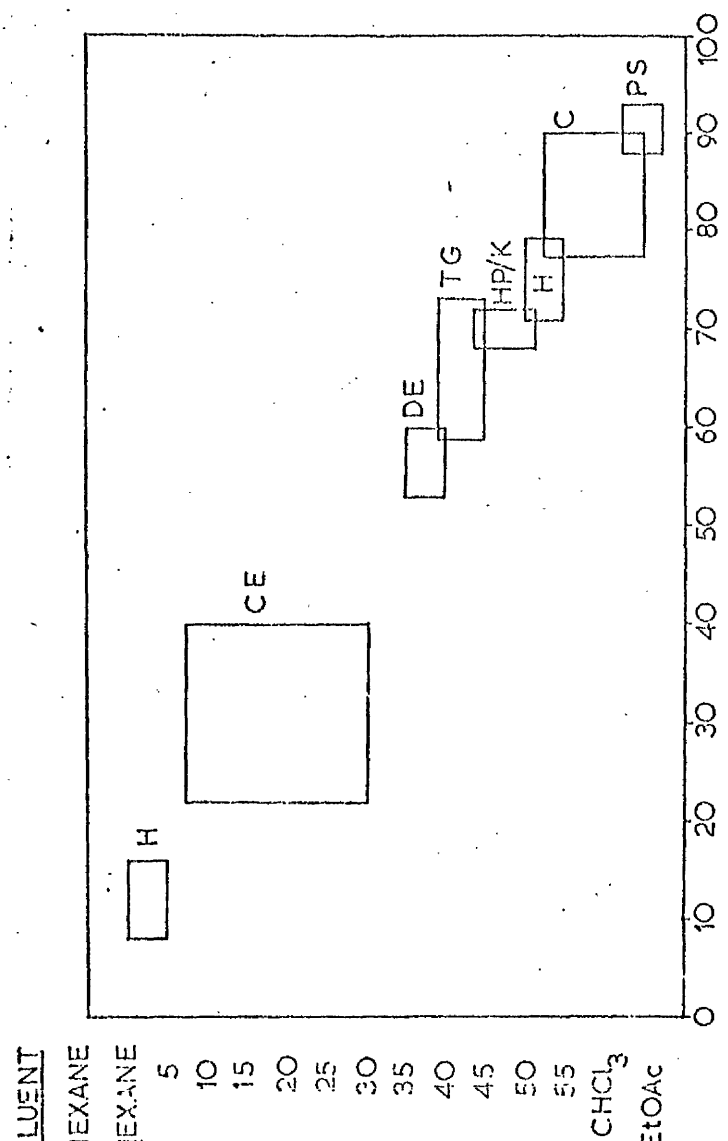


Fig.3.6(a) Group separations achieved on a straight-phase cholanyl LH-20 column (500 X 4mm i.d.) eluted with benzene, of a total lipid extract derived from a human aorta. For comparison purposes, Fig.3.6(b) overleaf illustrates the chromatography of this extract on a silicic acid column.



H: HYDROCARBONS
CE: CHOLESTEROL ESTERS
DE: 26-HYDROXYCHOLESTEROL DIESTERS
TG: TRIGLYCERIDES
HP: HYDROPEROXY ESTERS
K: KETO ESTERS
H: HYDROXY ESTERS
C: CHOLESTEROL
PS: POLAR STEROLS

Fig.3.6(b) Typical elution pattern obtained during silicic acid column chromatography of lipids from a severely diseased aorta.

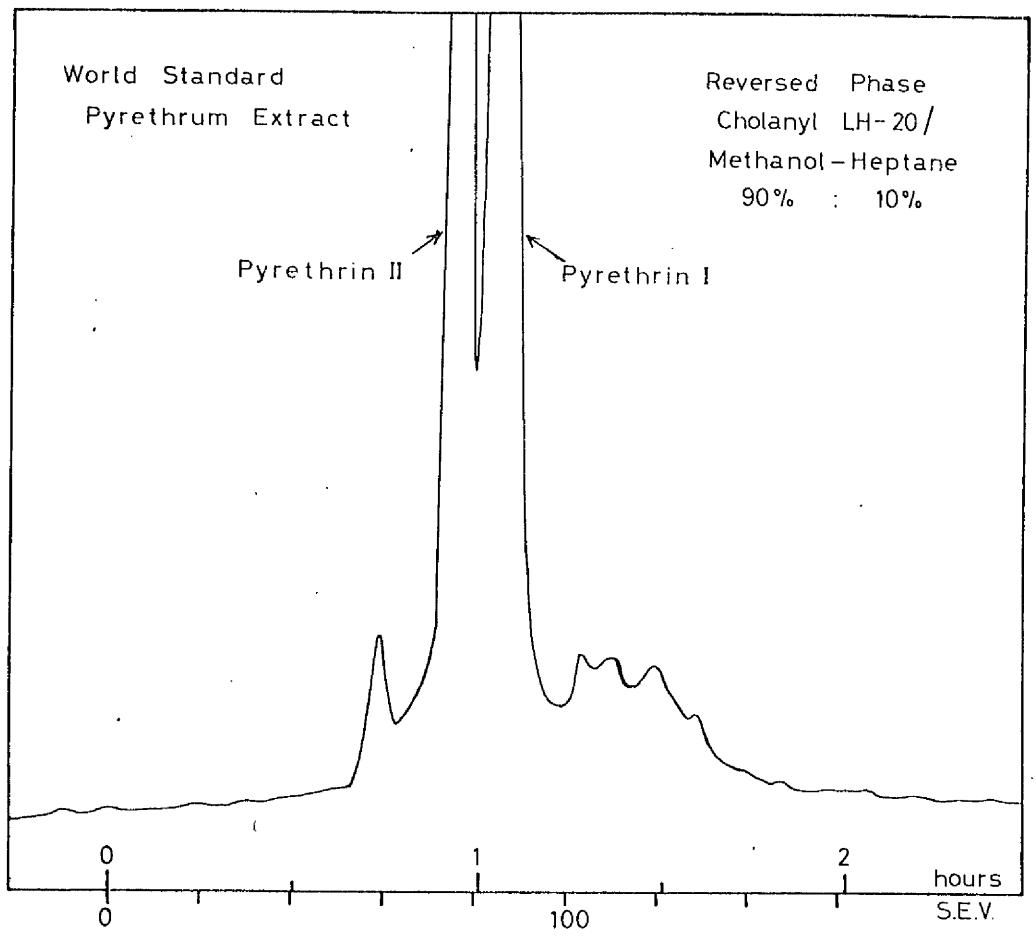


Fig.3.7 Chromatography of a World Standard pyrethrum extract on a reversed-phase cholanyl LH-20 column (700 X 1.6 mm i.d.) eluted with methanol-heptane (9:1), showing the partial separation of the similar compounds, pyrethrin I and pyrethrin II. Pyrethrin II contains an additional methoxycarbonyl function which results in its earlier elution.

However, no such effect has been found with fully lipophilic gels[90,92] or with hydroxy-5β-cholanyl substituted LH-20.

The pattern of elution in the reversed-phase system follows the order: acids, alcohols, hydrocarbons. Lipophilic character is an important factor in determining elution volumes. Thus the presence of a hydrocarbon side-chain on the steroid nucleus causes longer retention of C_{27}, C_{28} and C_{29} sterols relative to steroids lacking the side-chain. The introduction of one or more double bonds to cholestanone, in conjugation with the carbonyl function decreases the SEV. Methylation of an acid increases its SEV.

The influence of molecular size on elution volumes is illustrated by the data for the n-alcohol series. In the straight-phase system, elution is in the order of decreasing molecular weight, while in the reversed-phase system the order is inverted. The separation of n-alcohols on a reversed-phase narrow-bore column is illustrated in Fig. 3.5, representing the liquid chromatograph trace with background noise removed. The straight-phase system is useful for group separation of mixtures while reversed-phase systems can, in addition, often resolve similar compounds within a group. In the former system, care must be taken, in quantitative work, to allow for partial resolution, as the composition may change between the start and end of the peak. Fig. 3.6 (a) shows group separations achieved on a straight-phase cholanyl LH-20 column (500 x 4 mm ID) eluted with benzene, of a total lipid extract derived from a human aorta. For comparison, Fig. 3.6(b) illustrates schematically chromatography of a similar extract on a classical silicic acid column.

Fig. 3.7 illustrates a chromatogram of a World Standard Pyrethrum

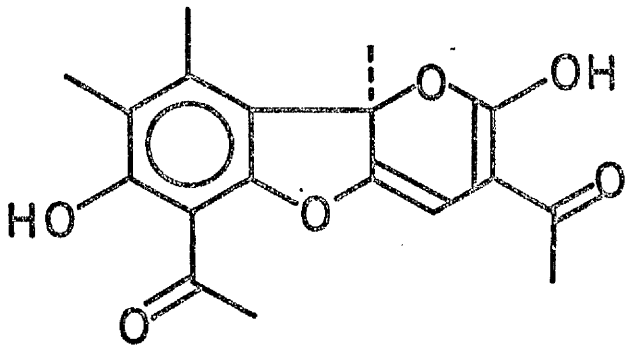
extract on a reversed-phase column, eluted with methanol-heptane (9:1). A partial separation has been achieved between the similar compounds, pyrethrin I and pyrethrin II. Pyrethrin II contains an additional methoxycarbonyl function which results in its earlier elution. Fig. 2.16 illustrates a two-dimensional chromatogram of the pyrethrum extract obtained by collecting the column eluant on a TLC plate and then developing the plate in the normal manner. The distinctive colour reaction of the two pyrethrins with ceric ammonium sulphate (green for pyrethrin I and purple for pyrethrin II) and the R_f values on TLC permitted the easy recognition of the compounds.

The gel was examined in both straight- and reversed-phase systems for ability to separate enantiomeric pairs and also pairs of phytosterol diastereoisomers. No success was apparent with any of the sets of compounds used. While a small separation was observed, for example, between cholesterol and campesterol, none could be detected between campesterol and the 24-epimer, dihydrobrassicasterol. To confirm that not even partial resolution of enantiomers was obtained, a racemic mixture was chromatographed and fractions were collected during elution. Usnic acid was chosen as a test substance because of its extremely high molecular rotation ($[\alpha]_D^{20}$ of each isomer is 503°, ref. 161), because it has a high extinction coefficient for the absorption band at 285 nm in the UV spectrum, and because both enantiomers were available commercially. In addition, the material is of interest as a natural product. The elution profile of the enantiomeric mixture, obtained by plotting the UV-absorption intensity versus elution volume, was indistinguishable from that obtained for D- or L-usnic acid. The optical rotation of the material in each half of the peak was measured and found to be zero.

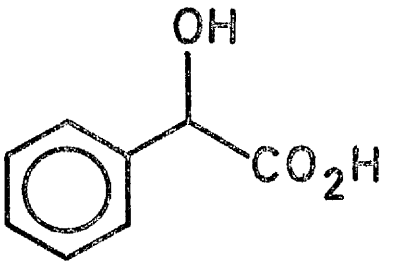
The absence of a chiral effect may be explained by a consideration of the solute-gel interaction. Separations on a gel column are based primarily on differences in the partition coefficients of the solute molecules between the mobile phase and the stationary phase. In addition, there is evidence that the ether linkages in the substituted gel provide sites for adsorption (see Chapter 5), but whether these are on the matrix directly or on an associated solvent layer must depend on whether the solute can displace the solvent molecules. The absence of a chiral effect indicates that the interaction of solutes with the steroidal hydrocarbon residues is insufficient to lead to significant differences in the partitioning of enantiomers and of the phytosterol diastereomers between the mobile and stationary phases.

3.4 Chromatography with chiral eluants.

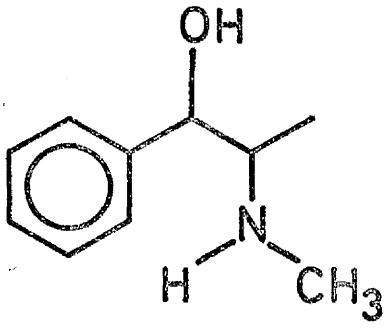
The possibility of resolving enantiomers by formation of diastereoisomers prior to chromatography has already been discussed. The alternative method, by direct chromatography, also depends on the formation of diastereomeric association complexes between solute and stationary phase. Also, it has already been noted that in liquid chromatography the mobile phase plays a much more important role than in gas chromatography, in view of the solute-solvent interactions and mobile-phase/stationary phase interactions. It therefore seemed likely that solute-solute interactions might occur also. If, instead of using a pure solvent or mixture of pure solvents as eluant, a solution was used of a suitable optically active material, then it is possible that solute-solute interactions might lead to diastereomeric association complexes in solution (in contrast to those formed on the surface of an optically active stationary phase) which might



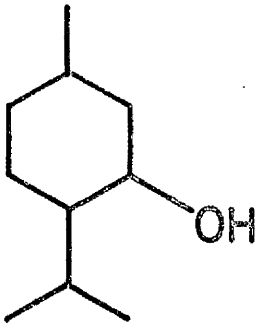
Usnic acid



Mandelic acid

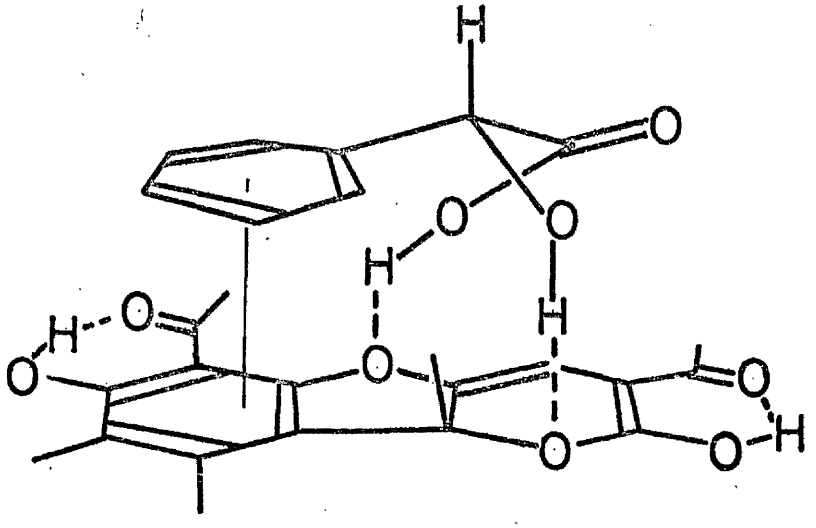


Ephedrine



Menthol

(a)



(b)

Fig.3.8 Substrates and resolving agents for the attempted separation of racemic mixtures using a chiral eluant.

be separable on a partition chromatography system. If the latter were also chiral, the likelihood of success would appear greater.

Accordingly, two systems were tried to test this principle.

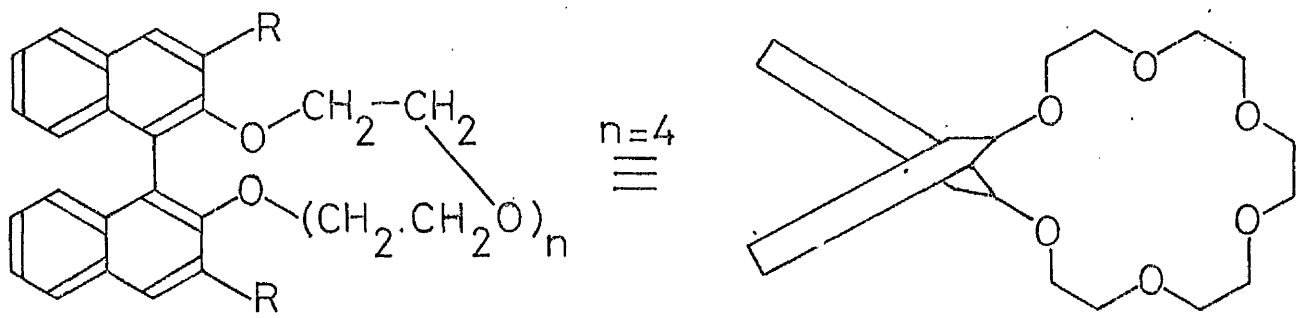
(i) Attempted Resolution of (±)-Usnic Acid on a Column Eluted with (-)-Mandelic Acid in Benzene.

These two compounds are illustrated in Fig. 3.8(a). One possible association complex between them is shown in (b): an analogy may be drawn to some extent between the three-point association here and that obtained in other systems described earlier. π-Electron interactions are known to occur between phenyl rings, and this is indicated by a solid line. Hydrogen bonds are marked by broken lines. The infrared spectrum of usnic acid alone and that of each enantiomer in the presence of (-)-mandelic acid in CCl_4 solution was examined for any sign of complex formation but none was observed.

A straight-phase column of hydroxy-5β-cholanyl LH-20 was equilibrated with a saturated solution of (-)-mandelic acid in benzene. (±)-Usnic acid (2 mg) was added to the column dissolved in 80 µl of the eluant and chromatographed in the normal manner. Fractions were collected and examined for partial resolution by the method described above (para 3.3). No sign of separation could be detected.

(ii) Attempted Resolution of (±)-Ephedrine on a Column Eluted with (-)-Menthol in Benzene.

Usnic acid was found to undergo solvolysis in the reversed-phase (MeOH/heptane 9:1) system and also it appeared to react with ephedrine or menthol when this was tried as a component in the straight-phase system. It was decided to try using (-)-menthol as the eluant component with racemic ephedrine as the solute. The structures of these compounds are



$$R = CH_2OCH_2.CO_2H$$

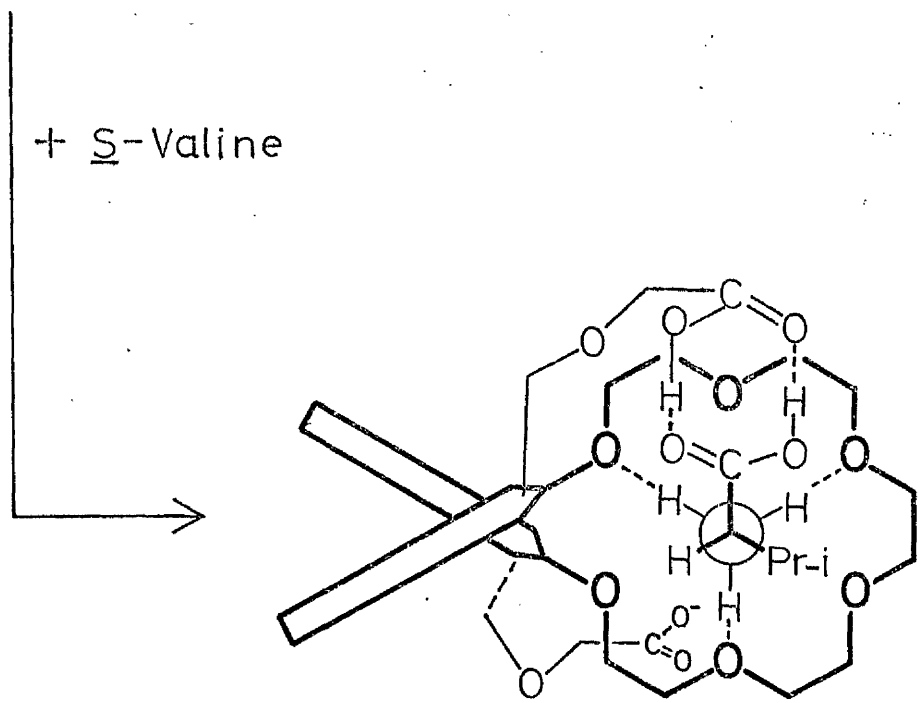


Fig.3.9 Cyclic polyether resolved by liquid-liquid chromatography through formation of a complex with a guest (S-valine) molecule.

illustrated in Fig. 3.8(a). Menthol is devoid of functionality apart from the hydroxyl group. The chiral centres are bonded mainly to hydrocarbon moieties, rather similar to the hydroxy-5β-cholanyl residue itself. The use of a volatile solid, menthol, as resolving agent also gave the advantage that the transport detector could be used. Solid menthol deposited on the wire was found to vaporise in the drying oven of the chromatograph (Fig. 2.14(a)).

Chromatography was carried out as for (i) above, but once again, no sign of resolution could be detected.

The failure of this approach in this instance cannot be explained with certainty. It may be that both isomers of the racemic mixture were able to form complexes with the resolving agent with equal facility, in view of the number of functional groups present, for example, in usnic acid, or that the partition chromatography system was not selective enough to distinguish between such complexes. The number of theoretical plates obtained with the straight-phase column (measured for cholesterol when benzene was the eluant) was 3100. This is much lower than the number required for resolution of diastereoisomers by GLC, which was measured at approximately 15,000.[43,162]

Similar approaches to the problem of enantiomer resolution have recently been published.[163-165] In some cases, complete success has been achieved. Thus Helgeson and co-workers have separated racemic cyclic polyethers of the type shown in Fig. 3.9 by liquid-liquid chromatography using a stationary phase consisting of a solution of (S)-valine in a mixture of acetic acid, water and benzene (40:10:15) supported by Celite.[164] The ability of the polyether host to form an association complex with a guest (valine) molecule determines the partition coefficient of the host

in the stationary phase. An examination of molecular models of the compounds led to the prediction that the cyclic polyether with n=4 would show "chiral recognition" of valine and that (RR) or (SS) complexes would be more stable than (RS) complexes. These predictions were borne out in practice. The structural requirements for efficient hydrogen bonding impose a high degree of selectivity on the guest molecule. Once again the ability to form several points of bonding is critically important.

A further example of (partial) resolution by partition was published by Bowman and co-workers,[166] who observed partial separation of enantiomers of mandelic acid and phenylephedrine in a 2-phase system, one phase of which was aqueous and the other an optically active ester of d-tartaric acid. At least two sites of hydrogen bonding were required of the substrate and resolving medium. Thus while the above-named compounds could be partially resolved, epichlorhydrin or monools could not be resolved at all. The work by Helgeson et al. is of interest as it indicates that a more sophisticated system is capable of achieving the separations which could not be obtained by the simpler approach described above.

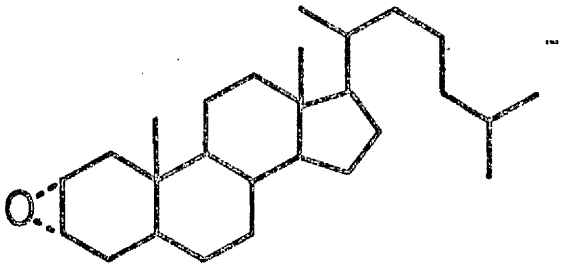
Chapter 4. HYDROXYALICYCLIC DERIVATIVES OF SEPHADEX LH-20.

4.1 Introduction.

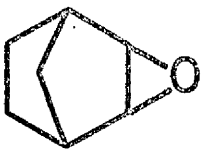
Further derivatives of LH-20 were prepared using alicyclic olefin oxides. By so doing, it was intended to decrease the chain length between the gel matrix and the chiral centre. The steric interaction between substituents would increase and confer a greater conformational preference on the gel-substituent link and therefore impart more rigidity to the system as a whole. It was hoped that this might give rise to a greater chiral effect by the substituents in the determination of the partition coefficients of solutes passing through the gel column.

Two problems were anticipated in the preparation of alicyclic oxide derivatives. Firstly, steric hindrance during the substitution process was expected to reduce the rate of reaction. The terminal aliphatic oxides which had previously been used were potentially less hindered as attack could occur at the ω-carbon atom. Secondly, if complex alicyclic oxides were chosen for the acid-catalysed substitution, the possibility was recognised that rearrangement reactions might also occur, leading to by-products which would not react with the gel (see for examples refs. 140-143). The simplest alicyclic oxide, cyclohexene oxide, was chosen as a model compound for the reaction of more complex oxides. Although not optically active, it had the advantage of being commercially available.

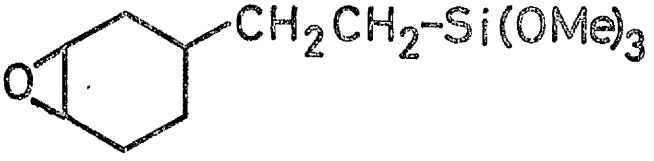
Four other alicyclic oxides were used, two of which were prepared from readily available optically active natural products, and the other two were of commercial origin (Fig. 4.1). 2α,3α-Oxido-5α-cholestane was prepared from cholesterol. This oxide was chosen because it satis-



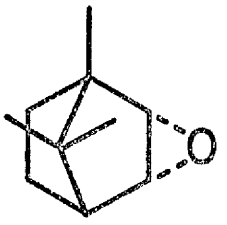
2α,3α-Oxido-5α-cholestane



exo-2,3-Oxidonorbornane



β-(3,4-Oxidocyclohexyl)-ethyltrimethoxy-silane



endo-2,3-Oxidobornane

Fig.4.1 Oxides used for the preparation of hydroxyalicyclic derivatives of Sephadex LH-20.

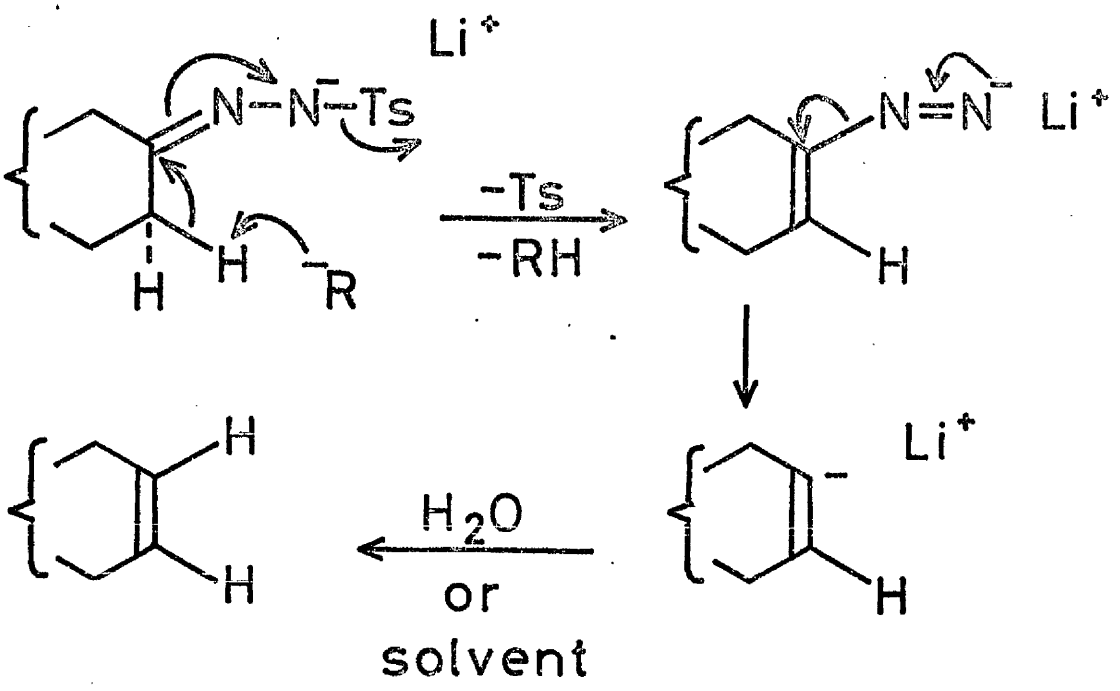
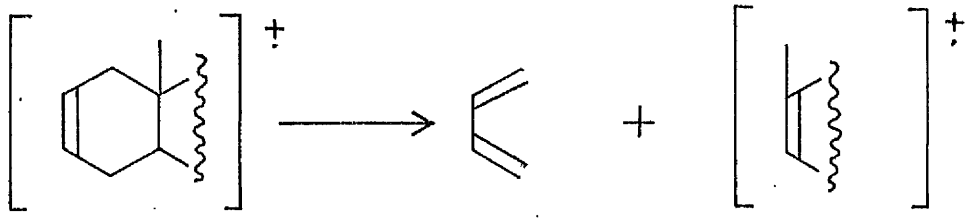


Fig.4.2 Proposed mechanism for the reaction of N-tosylhydrazones with alkyllithium reagents.

fied the criteria discussed in Chapter 3 for 23,24-oxido-5β-cholane. Also, the 2α,3α-oxide is comparatively unhindered and is furthest from any ring junction. The preparation of the olefin from cholestanone was accomplished by reaction of the tozylhydrazone with n-butyllithium.[116,167-169] The method was first described by Shapiro,[167] who suggested a mechanism involving an intermediate carbanion, Fig. 4.2 (see also ref. 168). It gives the least-substituted olefin in high yield. The spectral data of the olefin were compared with literature values[170] and appeared to be consistent. The position of the double bond was also indicated by the mass spectrum, which showed a major fragment corresponding to the product of a retro-Diels-Alder reaction and which was identical to that of a commercial standard of 5α-cholest-2-ene.



$M^+ = 370$ m/e = 316

The oxide prepared from this olefin by reaction with per-acid has been shown to be the α-oxide.[117]

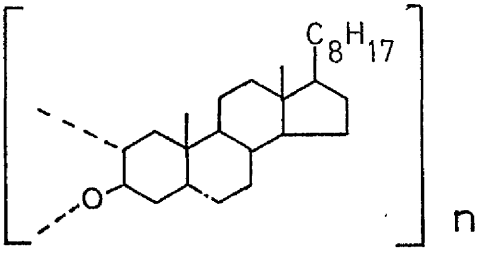
Endo-2,3-oxidobornane was prepared from α-pinene. This oxide was known to undergo rearrangements,[119] but following the opening of the oxide ring in the substitution reaction, the hydrocarbon skeleton of bornane would be stable. Exo-2,3-oxidonorbornane was commercially available and was chosen for comparison with oxidobornane. Also available commercially was β-(3,4-oxidocyclohexyl)-ethyltrimethoxysilane, which was of interest due to the presence of the silyl group.

4.2 Preparation of the Gels.

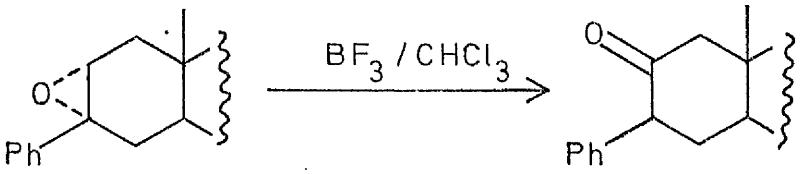
The reaction between LH-20 and cyclohexene oxide was initially

carried out under the conditions used for the preparation of cholanyl LH-20. Using 1 equivalent of oxide per equivalent of LH-20 (here considered as a tribasic acid in view of the three free hydroxyl groups per monomer unit of the gel matrix), the degree of substitution was only 19% by weight, compared to 40% by weight for the oxidocholane reaction. This is ascribed to the effects of steric hindrance and the occurrence of side-reactions. When a large excess of reagent was used (5 equivalents of oxide per equivalent of gel), a product was obtained containing 37.5% by weight of substituent (2 hydroxycyclohexyl residues per sugar ring of the dextran).

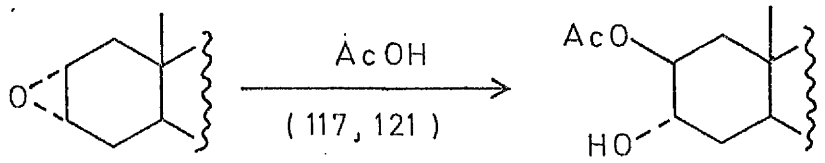
The other oxides showed a similar decrease in the efficiency of incorporation. Thus oxidocholestane yielded an initial product containing 16% by weight of substituent, which was increased on repeating the reaction by a similar amount. Approximately 20% of the oxide was incorporated in each step. The remainder gave a polymeric material, presumably of the type:



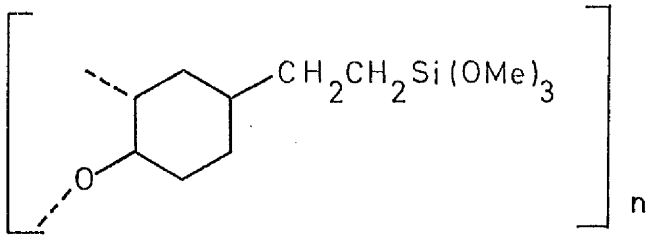
Cookson and Hudec reported the rearrangement of 2α,3α-oxido-3β-phenyl-5α-cholestane with BF_3 etherate in non-basic solvents, for example $CHCl_3$, to give 2-keto-3α-phenyl-5α-cholestane.[120] Examination of the soluble residues from the gel reaction by IR spectroscopy showed that little ketonic material was formed. The phenyl group would clearly affect the mode of reaction of an oxide by stabilising an intermediate carbonium ion.



The direction of opening of the oxide during the gel reaction, by analogy to its reaction with acetic acid,[117,121] would be diaxial to give a 3α-hydroxyl group:



Polymeric material was obtained also in the reaction of β-(3,4-oxidocyclohexyl)-ethyltrimethoxysilane with LH-20. Once again the structure was not established but was possibly of the type:



It appears likely, however, that the silyl moiety might also be involved in the polymerisation. This oxide is used in industry as a silane coupling reagent in the preparation of composite materials such as glass fibre reinforced plastics.[171] The silane is sensitive to moisture and reacts with materials containing free hydroxyl groups (Fig. 4.3) including silicates in glass. Contact with atmospheric moisture causes slow hydrolysis and polymerisation. However, under the experimental conditions used, care was taken to exclude moisture. From the reactions outlined in Fig. 4.3 it can be seen that catalytic amounts of water only are needed, and it is always possible that traces of moisture were present. In view of the chromatographic properties of the gel

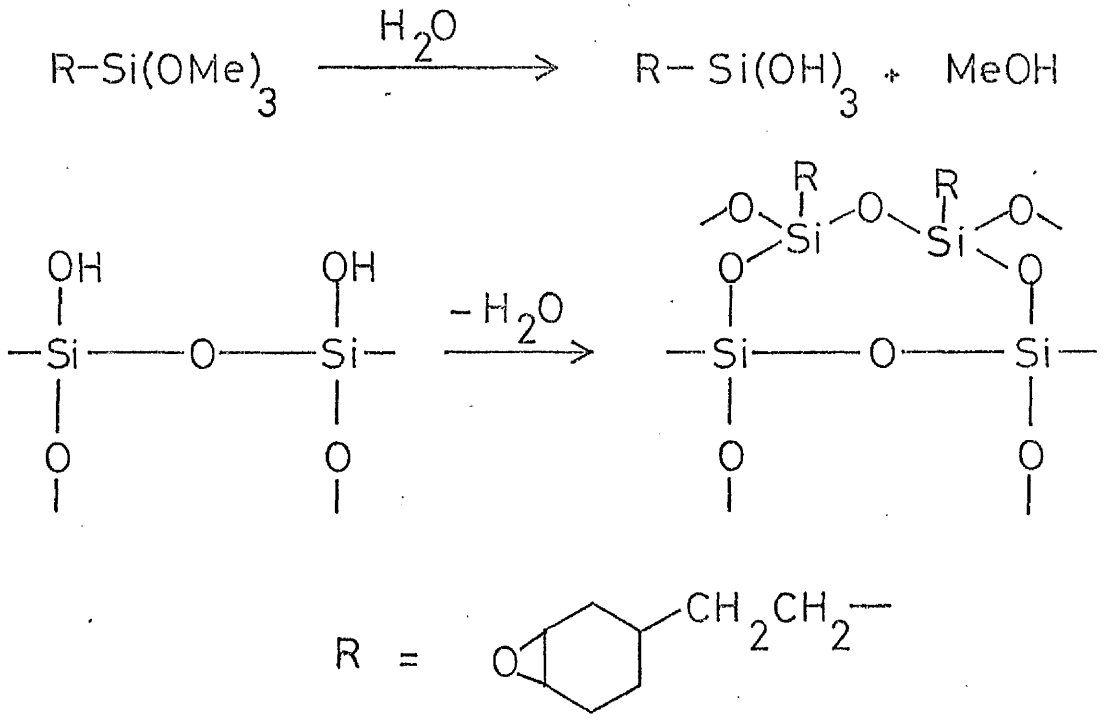
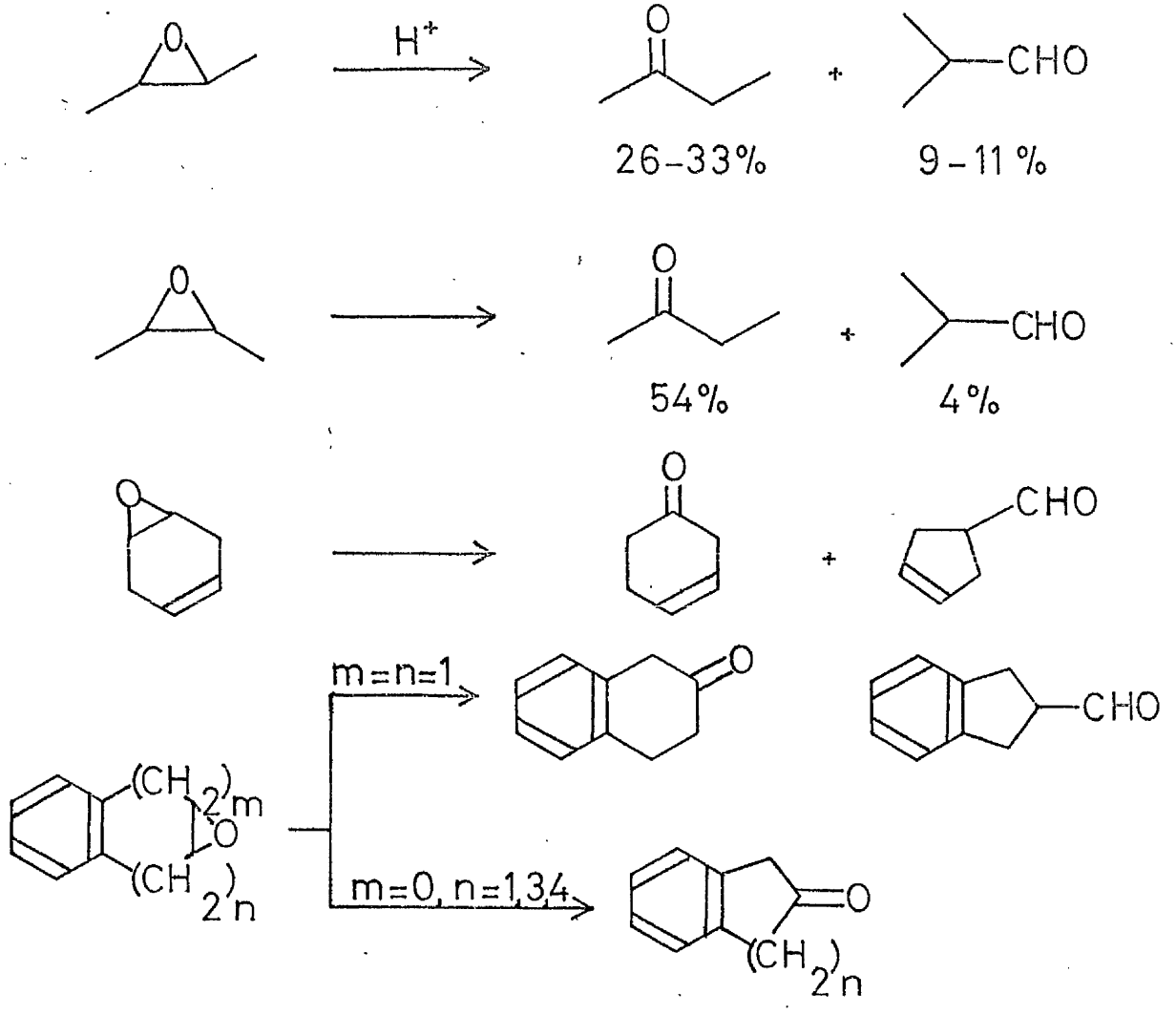


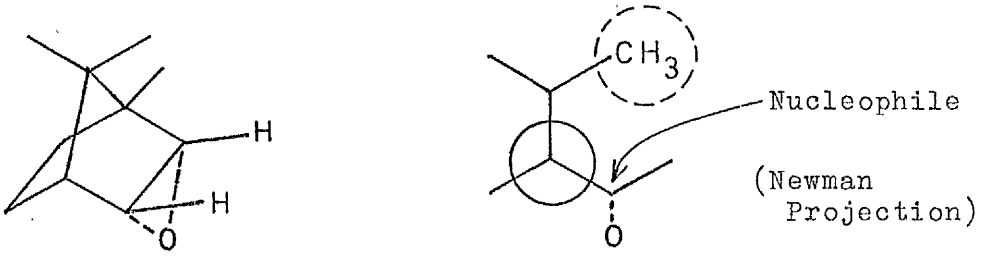
Fig.4.3 The reaction of a silane coupling reagent with silicates in glass.

Fig.4.4 Examples of acid-catalysed rearrangement reactions of disecondary epoxides.



prepared with this oxide (see para. 4.3 below) and also because of the failure of the product to react further with more reagent, it was concluded that silane polymerisation had occurred.

Oxidonorbornane and oxidobornane both gave rise to rearrangement products in the presence of BF_3. The latter oxide had a very poor incorporation into the gel. Reference has already been made to the by-products, studied by Borowiecki and Chrétien-Bessière[119] (see Fig. 2.9). The gem-dimethyl group above the plane of the cyclohexane ring may have caused the rearrangement reaction to proceed more readily than the substitution reaction.



Several examples are known of the rearrangement of disecondary epoxides (Fig. 4.4). The simplest of these, 2,3-oxidobutane, has been observed by House to show some stereospecificity.[172] Thus the trans-oxide gives an appreciable amount of iso-butyraldehyde. Cyclohexadiene mono-oxide also undergoes alkyl migration, with simultaneous ring contraction, to give an aldehyde.[173] Epoxides derived from indene,[174] 1,2- and 1,4-dihydronaphthalene,[175] 3,4-benzo-1,3-cycloheptadiene[176] and 3,4-benzo-1,3-cyclooctadiene[177] all undergo rearrangement to ketones in the presence of the Lewis acid, $MgBr_2$.

4.3 Properties of the Gels.

The gels prepared from alicyclic oxides were stable and appeared to have similar physical properties to hydroxy-5β-cholanyl LH-20. Solvent regain values and SEV data only were examined. It was assumed

TABLE 4.1

Solvent Regain Values for Hydroxycyclohexyl-Substituted LH-20.

Solvent	Solvent Regain Value (a)	(b)
Heptane	0.25	0.38
Cyclohexane	0.44	0.57
Carbon tetrachloride	2.05	1.29
Toluene	1.08	1.25
Benzene	1.11	1.27
Tetrahydrofuran	1.27	0.91
Chloroform	2.44	1.65
Methylene chloride	1.91	1.44
Acetone	0.47	0.59
Methanol	0.63	0.79

Determined according to Helfferich.[128]

(a) g of solvent taken up by 1 g dry gel.

(b) ml of solvent taken up by 1 g dry gel.

TABLE 4.2

Values of Standard Elution Volume (S.E.V.) for Hydroxycyclohexyl LH-20 in the Straight-Phase System (Benzene).

Compound	Mol. Wt.	S.E.V.
5α-Cholestane	372	51.8
5α-Cholestan-3-one	386	55.8
5α-Cholestan-3β-ol	388	88.4
Cholesteryl chloride	404.5	55.6
Cholesteryl acetate	428	52.3
Cholesteryl benzoate	490	50.5
Cholesteryl palmitate	624	44.5
4-Cholesten-3-one	384	56.6
5-Cholesten-3-one	384	55.3
Cholesterol	386	94.4
Coprostanol	388	86.6
Stigmasterol	412	90.8
β-Sitosterol	414	91.6
Dihydrobrassicasterol	400	93.1
Lanosterol	426	79.4
4-Cholestene-3, 6-dione	398	56.9
4,6-Cholestadien-3-one	382	58.7
24-Chloro-5β-cholane	364.5	58.0
5β-Cholan-24-ol	346	106.7
7-Ketocholesteryl acetate	442	53.0
Progesterone	314	62.9

TABLE 4.2 (Cont'd).

Compound	Mol. Wt.	S.E.V.
5-Pregnen-3β-ol	302	109.6
(±)-Usnic acid	344	64.7
n-Dodecan-1-ol	186	108.2
n-Tetradecan-1-ol	214	101.2
n-Hexadecan-1-ol	242	95.2
n-Octadecan-1-ol	270	89.7
n-Eicosan-1-ol	298	84.9
n-Docosan-1-ol	326	80.2
n-Tetracosan-1-ol	354	76.9
n-Hexacosan-1-ol	382	73.6
Tristearin	890	41.2

TABLE 4.3

S.E.V. Data for Hydroxycyclohexyl LH-20 in the Reversed-Phase System (Methanol-Heptane 9:1 v/v).

Compound	Mol. Wt.	S.E.V.
5α-Cholestane	372	193
Cholesteryl acetate	428	141
5α-Cholestan-3-one	386	118
5α-Cholestan-3β-ol	388	115
Cholesterol	386	118
β-Sitosterol	414	125
Stigmasterol	412	122
n-Tetradecan-1-ol	214	87
n-Hexadecan-1-ol	242	92
n-Octadecan-1-ol	270	97
n-Eicosan-1-ol	298	103
n-Docosan-1-ol	326	109
n-Tetracosan-1-ol	354	116

TABLE 4.4

S.E.V. Data for Epimeric Hydroxysteroids and Δ^5/5α-Steroids in the Hydroxycyclohexyl LH-20 Straight-Phase System (Benzene).

Compound	Mol. Wt.	S.E.V. (a)	S.E.V. (b)	OH Conformation
5α-Cholestan-3β-ol	388	88.4	101	equatorial (e)
5α-Cholestan-3α-ol (Epicholestanol)	388	82.9	90.5	axial (a)
5β-Cholestan-3α-ol (Epicoprostanol)	388	87.0		e
5β-Cholestan-3β-ol (Coprostanol)	388	87.0		a
5-Cholesten-3β-ol (Cholesterol	386	94.4	103	e
5-Cholesten-3α-ol (Epicholesterol)	386	70.1	76.5	a
3β-Hydroxy-5α-androstan-17-one	290	114	101	e
3α-Hydroxy-5α-androstan-17-one	290	104	93.8	a
3α-Hydroxy-5β-androstan-17-one	290	107	97.2	e
3β-Hydroxy-5β-androstan-17-one	290	104	93.0	a
3α-Hydroxy-5β-pregnan-20-one	318	99.5	93.2	e
3β-Hydroxy-5β-pregnan-20-one	318	99.5	93.2	a
5-Cholestene-3β, 7β-diol	402	170	117	e
5-Cholestene-3β, 7α-diol	402	162	106	a
5β-Cholan-7β-ol	346	67.0		e
5β-Cholan-7α-ol	346	67.0		a
11α-Hydroxy-pregn-4-ene-3,20-dione	330	115	99.0	e
11β-Hydroxy-pregn-4-ene-3,20-dione	330	108	92.4	a

TABLE 4.4 (Cont'd).

Compound	Mol. Wt.	S.E.V. (a)	S.E.V. (b)	OH Conformation
5α-Androstan-17β-ol	276	103	106	Ψ- e
5α-Androstan-17α-ol	276	95.2	97.2	Ψ- a
5-Pregnene-3β, 20β-diol	318	170	161	more hindered
5-Pregnene-3β, 20α-diol	318	183	174	less hindered
5-Cholestene-3β, 24-(RS)-diol 3β-acetate	444	69.4	67.3	mobile
3β-Hydroxy-5α-androstan-17-one	290	101		
3β-Hydroxy-5-androsten-17-one	288	108		
17α-Methyl-5α-androstane-3β, 17β-diol	306	201		
17α-Methyl-5-androstene-3β, 17β-diol	304	221		
3β-Hydroxy-5β-pregnan-20-one	318	97.2		
3β-Hydroxy-5-pregnen-20-one	316	105		
5α-Pregnane-3β,20β-diol	320	193		
5-Pregnene-3β,20β-diol	318	211		
Cholestanyl acetate	430	52.3		
Cholesteryl acetate	428	52.3		
Cholestane	372	51.8		
5-Cholestene	370	51.8		

(a) Hydroxycyclohexyl substituted Sephadex LH-20.

(b) Nedox 1114 substituted Sephadex LH-20.

that the recovery of material would be high for these gels. Elution profiles, obtained directly on the liquid chromatographs, were symmetrical and "tailing" peaks were not observed. The gel prepared from the model reagent, cyclohexene oxide, was evaluated in more detail than the other gels and will be considered separately.

4.3.1 Hydroxycyclohexyl LH-20 (containing 37.5% by weight of substituent).

Solvent regain values are listed in Table 4.1 and SEV data in Tables 4.2 - 4.4. The gel swelled in solvents of widely different polarity, the highest values being obtained with halogenated and aromatic solvents. Absolute values are lower than for cholanyl LH-20 and are similar to Nedox 1114 LH-20 of approximately the same degree of substitution, inasmuch as data are available.[90] Both of these gels swell to a greater extent in heptane than does hydroxycyclohexyl LH-20 (taking the SRV values relative to benzene). The nature of the substituent must in part be responsible for the affinity of the gel for solvents. When solvent regain values are compared for a hydroxyalkyl gel containing a similar number of substituents, on average, per sugar ring, the difference is enhanced: the gel swells more in heptane than methanol (SRV of 0.8 and 0.3 respectively).

Straight-phase and reversed-phase systems were examined in an evaluation of the gel as a stationary phase for chromatography, using benzene or methanol-heptane (9:1 v/v) as eluting solvent, respectively. The order of elution of materials from the column was found to be similar to that obtained with cholanyl LH-20 in both systems.

In the reversed-phase system, the retardation of non-polar compounds relative to hydroxylic compounds is less than on cholanyl LH-20. Thus

the elution volume of cholestane (relative to cholesterol) is 2.7 on the cholanyl gel but only 1.6 on the cyclohexyl gel. A similar effect is obtained in hydroxyalkyl Sephadex systems. When the degree of substitution of the gel is low, non-polar compounds are not strongly retarded on the column. The effect may be explained in terms of the relative polarities of the gel phase and mobile phase. As the polarity of the gel is reduced (by increasing the weight of lipophilic substituent) non-polar substances favour the stationary phase over the mobile phase and the elution volume increases. The polarity of the cyclohexyl gel must therefore be greater than that of the cholanyl gel. Support for this idea can be taken from the solvent regain values of the two gels in polar and non-polar solvents. The cyclohexyl gel swells less in heptane and more in methanol than does the cholanyl gel. It appears that the polarity of the gel is determined by the degree of substitution by weight and by the size and shape of the substituent. On a molar basis (no. of substituents per sugar ring) the cyclohexyl gel is more highly substituted than the cholanyl gel (2 substituents as opposed to 0.6 per sugar ring). The chromatography system may be considered as tending towards a straight-phase one as the polarity of the gel increases. An intermediate point might be anticipated at which a gel filtration system is obtained (compare the gel filtration system obtained by adjusting the polarity of the solvent, ref. 92).

Eight pairs of steroids containing epimeric hydroxyl substituents at the C-3, 7, 11, 17 or 20 positions were totally or partially resolved on the straight-phase column. 24(R)- and 24(S)-hydroxycholesterol were not separable, and, with the exception of 3α- and 3β-hydroxy-5β-androstan-17-one, those epimeric sterols examined which possessed the 5β- configur-

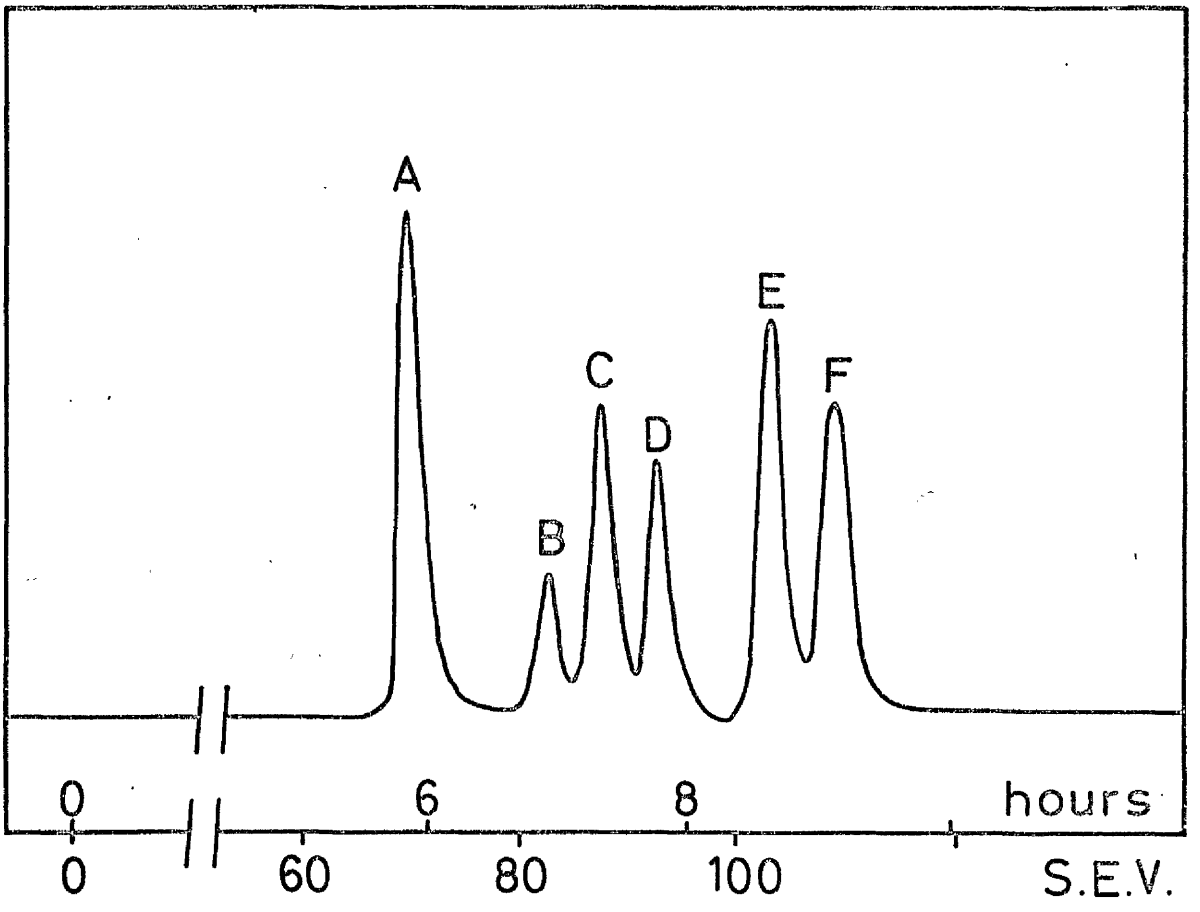


Fig.4.5 Separation of epimeric 3-hydroxysteroids on a straight-phase hydroxycyclohexyl LH-20 column (100 X 0.3 cm i.d.) eluted with benzene. A = Epicholesterol; B = Epicholestanol; C = Cholestanol; D = Cholesterol; E = Androsterone; F = Epiandrosterone.

ation (3 pairs) were also chromatographically indistinguishable in this system. [For comparison purposes, some of these epimeric pairs were chromatographed on Nedox 1114 gel (Table 4.4)]. Ellingboe and co-workers[90] have also achieved good separations of steroids containing epimeric hydroxyl groups on hydroxyalkyl LH-20 using other solvent systems. Thus 20α- and 20β-hydroxypregn-4-en-3-one and 11α- and 11β-hydroxypregn-4-en-3,20-dione were separated on a straight-phase column eluted with heptane-chloroform (8:2). However, Eneroth and Nyström obtained only poor separations of epimeric sterols on methylated Sephadex and LH-20.[128]

Five 3β-hydroxy steroids containing a double bond at the 5-position were found to be separable from the corresponding dihydro (5α) steroids. These separations were not observed on the Nedox gel. A small difference in the retention volume of cholestanol relative to cholesterol has been observed on methylated Sephadex in various solvents.[178] Fig. 4.5 illustrates the separation of six steroids on a 1 m straight-phase hydroxycyclohexyl column.

The process underlying the separation of epimeric sterols is presumed to be one of adsorption of the solute on the gel network. Hydrogen bonding provides a probable explanation of such binding and has been implicated by previous workers[92,145,154-157,179,180] in connection with retardation of hydroxylic solutes on both lipophilic and hydrophilic gel columns. The expected hydrogen bonding sites are the ether linkages in the gel and there is some evidence for their participation.[145,179]

In both 5α- and Δ^5-steroids, equatorial alcohols were retarded relative to their axial epimers. In the 5β- series, three out of

TABLE 4.5

Solvent Regain Values for Hydroxy-Alicyclic-Substituted LH-20.

Solvent	Solvent Regain Value * (a)	(b)	(c)	(d)
Cyclohexane	1.04(1.33)	0.44(0.56)	0.24(0.31)	0.40(0.51)
Benzene	1.38(1.59)	0.57(0.66)	0.86(0.99)	0.84(0.97)
Chloroform	2.60(1.76)	0.88(0.59)	2.41(1.63)	3.26(2.20)
Methanol	0.85(1.08)	0.61(0.77)	1.12(1.42)	1.68(2.13)

* Determined according to Helfferich:[128] values are for g of solvent taken up by 1 g of dry gel and, in parenthesis, ml per g of dry gel.

(a) = hydroxycholestanyl gel (37.5% by weight of substituent).

(b) = (trimethoxysilylethyl)-hydroxycyclohexyl gel (34% by weight of substituent).

(c) = hydroxynorbornyl gel (20.2% by weight of substituent).

(d) = hydroxybornyl gel (7% by weight of substituent).

four pairs of epimeric alcohols failed to separate, indicating that the ring skeleton must be involved in the solute-gel interaction. This is supported by the observed separations of Δ^5-steroids from the corresponding dihydro (5α) compounds. The olefinic 3-hydroxy steroids were retarded relative to the saturated compounds - probably because of their greater potentiality for hydrogen bonding. Neither cholest-5-ene nor cholesteryl acetate were separable in this system from their dihydro analogues : hydrogen bonding to the 3- position is absent in the hydrocarbons, and would be weak in the acetates. The S E V values of the 5β-cholan-7-ols are unusually low, implying that hydrogen bonding is hindered by the molecular geometry. Structural rigidity is an important factor: the markedly different S E V values of C-20 epimers reflect the distinctive steric requirements of their preferred conformations, whereas C-24 epimers failed to separate.

4.3.2 Other Hydroxyalicyclic Gels.

Solvent regain values are quoted in Table 4.5. As with the previous gels, swelling occurs in solvents of widely different polarity. Hydroxycholestanyl LH-20 is a less polar gel than hydroxycyclohexyl LH-20: the degree of swelling in methanol relative to cyclohexane is less, and in this respect is similar to cholanyl LH-20 and hydroxyalkyl LH-20. Trimethoxysilylethyl-hydroxycyclohexyl (TMSE-hydroxycyclohexyl) LH-20 is unusual in that it swells more in methanol than in chloroform (ml/g) and also because the degree of swelling over the polarity range is generally low and does not vary widely (compare, for example, the norbornyl gel where the swelling in chloroform is five times greater than in cyclohexane). Norbornyl and bornyl LH-20 are more polar than the cholestanyl gel, as expected from the degree of substitution.

TABLE 4.6

Standard Elution Volume (S.E.V.) Data for Hydroxy-Alicyclic-Substituted LH-20 Gels in the Straight-Phase (Benzene) System.

Compound	Mol. Wt.	S.E.V.* Cholestanyl gel	Norbornyl gel	Bornyl gel
5α-Cholestane	372	87.2	67.8	57.9
5-Cholestene	370	86.4	71.5	57.9
5α-Cholestan-3-one	386	98.5 (428)	74.9	56.5
4-Cholesten-3-one	384	102	78.4 (127)	66.8
Progesterone	314	98.5	90.0	83.5
Cholesteryl acetate	428	87.2	67.8 (142)	60.1
Cholesteryl butyrate	456	77.9	65.3	59.0
Cholesteryl palmitate	624	75.1	59.2	52.3
Tristearin	890	65.2	58.7	54.1
Cholestanol	388	171 (364)	112 (132)	88.6
Epicholestanol	388	140	104	80.8
Cholesterol	386	175 (353)	117 (135)	87.9
Epicholesterol	386	118 (305)	88.0 (136)	73.5
Stigmasterol	412	167 (371)	113 (135)	89.0
Poriferasterol	412	167 (371)	113 (135)	89.0
β-Sitosterol	414	164 (381)	108 (125)	94.2
Dihydrobrassicasterol	400		(122)	
Lanosterol	426	118	100	76.1
24-(RS)-Hydroxy-cholesteryl acetate	444	102	90.5	78.3
Epiandrosterone	290	159	176	186

* Figures in parenthesis are for the reversed-phase system (methanol-heptane 9:1 v/v).

TABLE 4.6 (Cont'd).

Compound	Mol. Wt.	S.E.V. Cholestanyl gel	Norbornyl gel	Bornyl gel
Dehydrocpiandrosterone	288	164	177	190
3α-Hydroxy-5β-pregnan-20-one	318	147	98.6	
3β-Hydroxy-5β-pregnan-20-one	318	140	98.6	
(+) Usnic Acid	344	93.5	90.0	74.6
(-) Usnic Acid	344	93.5	90.0	74.6

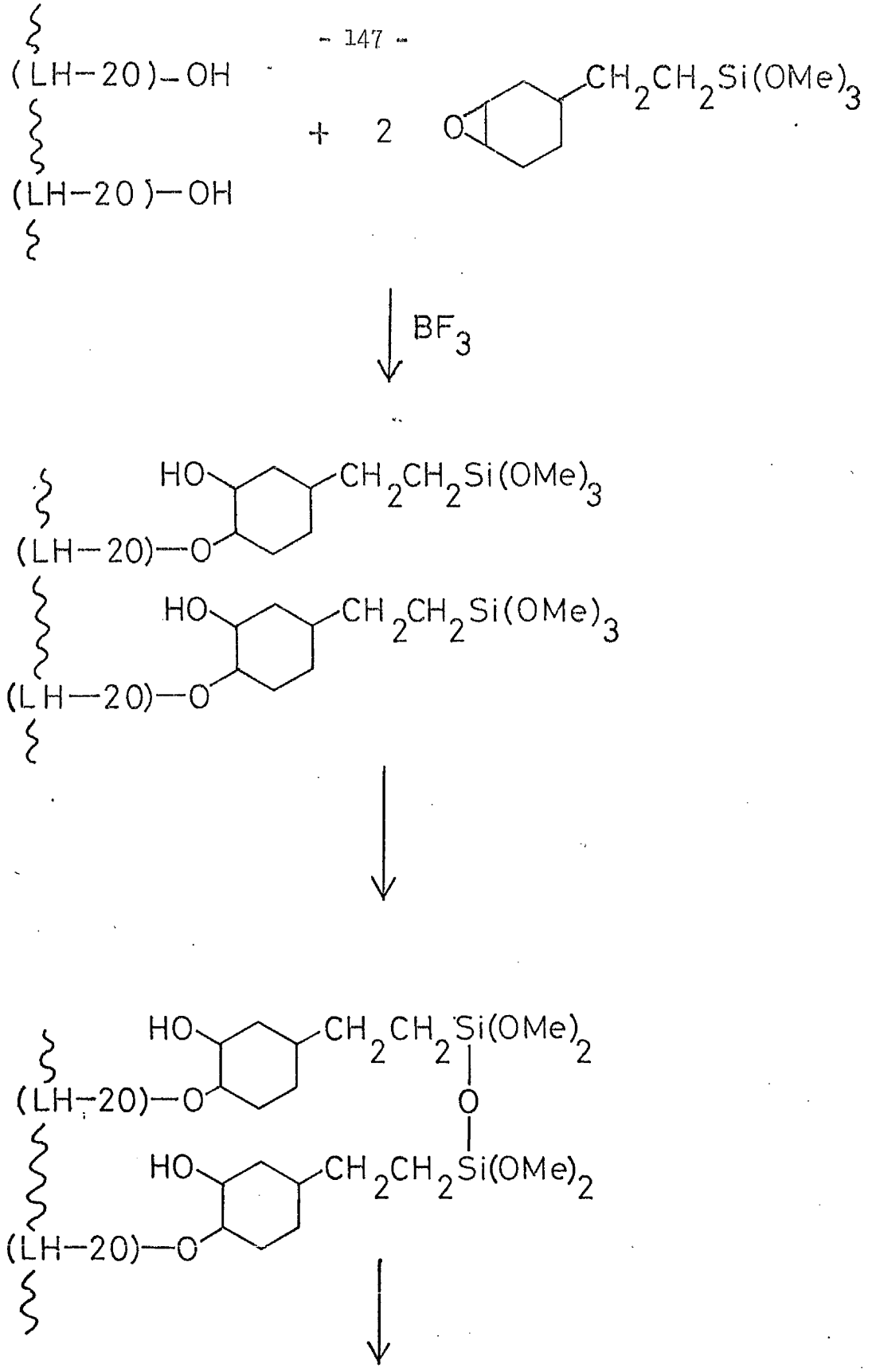


Fig.4.6 Postulated polymerisation of the silyl moieties during the preparation of TMSE-hydroxycyclohexyl LH-20. In the figure, (LH-20)—OH represents a monomer unit in the LH-20 matrix.

SEV data for a number of substances are given in Table 4.6. TMSE-hydroxycyclohexyl LH-20 appeared to be completely inert with respect to chromatographic properties. No separations were observed of materials varying both in size and polarity, in straight-phase or reversed-phase solvent systems (benzene or methanol-heptane 9:1): ephedrine, deoxycholic acid, cholesterol, cholesteryl palmitate and cholesterol TMS ether all had a standard elution volume of 55 in the benzene system. The variance of this behaviour from that of the other gels suggests that the silyl moieties have reacted during the substitution reaction (Fig. 4.6) to leave effectively an inert surface within the gel pores.

The order of elution of materials from the other gels is similar to that obtained with hydroxycyclohexyl LH-20. Attention was focused on three specific types of separation:

(1) the separation of epimeric hydroxylic steroids: 5α-cholestan-3β-ol and cholesterol were separable from their C-3 epimers. Also, 3α- and 3β-hydroxy-5β-pregnane-20-one were separable on hydroxy-5α-cholestanyl LH-20. The separation of epimeric 5β-steroidal alcohols represents an extension of the properties found previously for hydroxycyclohexyl LH-20. In addition, the separations of 5α-steroidal epimers were markedly increased on the cholestanyl gel.

(2) the separation of 3β-hydroxy-Δ^5/5α-steroids: cholesterol and dehydroepiandrosterone were distinguishable from the corresponding 5α-steroids although the degree of separation was less than that obtained on hydroxycyclohexyl LH-20.

(3) the separation of enantiomers and closely-similar diastereomers: (+)- and (-)-usnic acid were not separable on either of the chirally-

substituted gels or on the hydroxynorbornyl gel. Stigmasterol and poriferasterol, and 24(R)- and 24(S)-hydroxycholesteryl-3β-acetate were not separable on any of the gels.

An enhanced separation of epimeric compounds on hydroxy-5α-cholestanyl LH-20 gel compared to the hydroxycyclohexyl gel indicates a stronger solute-gel interaction in the former system. This may be due to the larger size of the alicyclic substituent in the steroid-substituted gel. However, the solute-gel interaction is insufficient to allow selective retention of enantiomeric or diastereomeric compounds, in agreement with earlier observations on hydroxy-5β-cholanyl LH-20.

Chapter 5. CONCLUSIONS.

5.1 An Assessment of Modified Dextran Gels.

In the foregoing chapters, several significant advances in lipophilic gel chromatography have been reported. These followed the early work by Sjövall and co-workers on modified Sephadex gels. Improvements have been made in the experimental conditions of the gel-substitution reaction, both in terms of the efficiency of olefin oxide incorporation and the amounts of reagent required. These are important points when the preparation of the oxide is difficult or time-consuming. Derivatives of Sephadex LH-20 have been prepared successfully from the chiral oxide, 23,24-oxido-5β-cholane, a primary olefin oxide with a very bulky substituent, and also from alicyclic oxides. It has been shown in the present work that disubstituted olefin oxides can be made to react with the gel. All the derivatives described above are homogenous with respect to the molecular weight of the substituent, unlike hydroxyalkyl LH-20 which is prepared from a mixture of olefin oxides. In addition, some improvements have been made in the types of separation which can be achieved and much has been learned in general about the behaviour of these lipophilic gels with respect to their chromatographic properties. One important observation was that the interaction of a chirally substituted gel with the range of compounds tried was not sufficiently selective to permit separation of enantiomeric compounds or of phytosterols diastereoisomeric in the side-chain.

The gels described in Chapters 3 and 4 are physically similar to hydroxyalkyl LH-20 and possess the desirable qualities of stability, capacity, mildness (of operating conditions) and adaptability to different types of system. Thus straight-phase and reversed-phase (and potentially,

also, gel permeation) systems are available. The first system is particularly useful for separating compounds differing in structure by the presence or absence, or the position and orientation, of hydroxyl groups. Although the elution volumes of compounds differing in polarity due to the presence or absence of other functions, for example carbonyl groups, are less well differentiated, separations can sometimes be achieved. A difference of 11 SEV units between the elution volume of cholestane (SEV 65) and cholest-4-en-3-one (SEV 76) on the hydroxy-5β-cholanyl LH-20 gel would permit their separation to be carried out on a column containing approximately 2000 theoretical plates (normally about 1 m long). Gel permeation effects, giving rise to separations on the basis of molecular size, are more prominent in straight-phase than reversed-phase systems. As the length of a hydrocarbon chain increases in, for example, a homologous series of n-alcohols the polarity simultaneously decreases. The effects of molecular size and polarity therefore tend to reinforce each other.

Reversed-phase systems can often be used for separating closely similar compounds differing in structure due to different hydrocarbon substituents. Carbonyl functions have a more pronounced effect on the elution volume in these systems. Thus saturated ketones, conjugated enones and conjugated dienones are separable (for example, cholestanone, cholest-4-en-3-one and cholest-4,6-dien-3-one have SEV values of 309, 248 and 199, respectively, on the hydroxy-5β-cholanyl gel).

Although the enantiomers and plant sterol diastereomers so far examined could not be distinguished, it is possible that other substances of these types might be separable. The question also arises as to whether other gels, containing different substituents would be

successful for this problem, or alternatively, whether the gels prepared would be able to separate these compounds under a different set of operating conditions or by varying the eluant composition. The mechanism of separation on the gels will now be discussed in an attempt to answer these questions.

5.2 Mechanism of Gel Separations.

In Chapter 1, the two types of separation process which generally occur in gel chromatography were discussed, namely gel permeation and gel partition chromatography. Following the experimental observations described in Chapters 3 and 4 concerning the retardation of hydroxylic compounds in straight-phase systems, or of non-polar compounds in reversed-phase systems, it is appropriate to add a third mode of separation deriving from interactions between the solute and gel, and termed "gel adsorption chromatography" by Determann.[136]

Adsorption effects have been noted in gel systems by several groups of workers (for a review, see ref. 148). The theory of gel permeation chromatography requires that all compounds be eluted between two exclusion limits (Fig. 1.8). When samples assume a partition coefficient and elution volume incompatible with GPC theory, an interaction of the solute with the gel phase must be occurring. Determann distinguishes three types of force responsible for abnormal sample retention on a column:[136] (a) Coulomb interactions between ions and charged species in the gel structure (ion exclusion or ion exchange); (b) Van der Waals interactions between the solute and the gel phase as a whole (partition); (c) adsorption of the solute on the polymer network of the gel. The last two forces have similar effects and are difficult to distinguish. Sjövall regards the transition

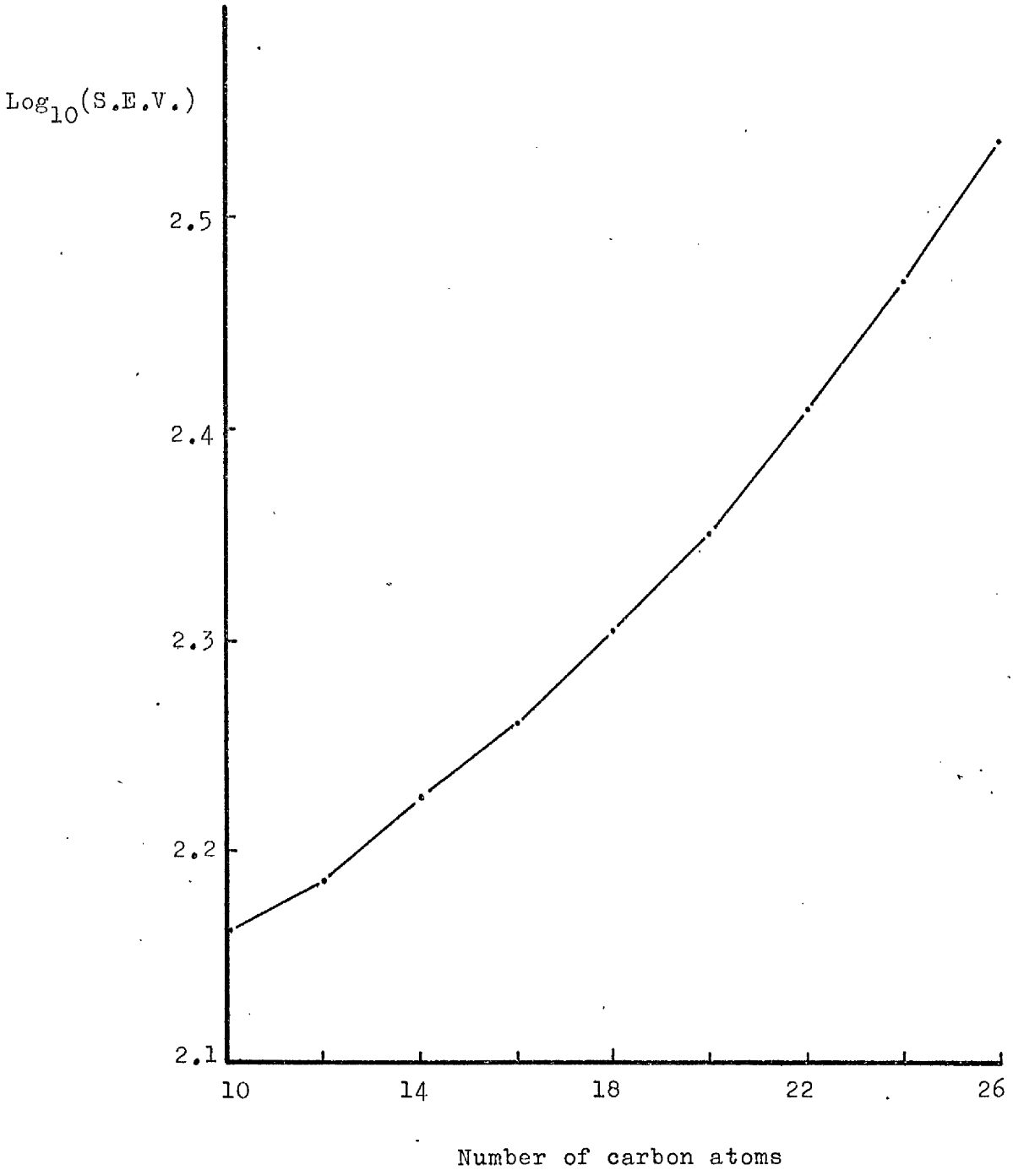


Fig.5.1 Graph of log(S.E.V.) versus the chain length for a series of n-alcohols chromatographed on a reversed-phase hydroxycholanyl LH-20 column.

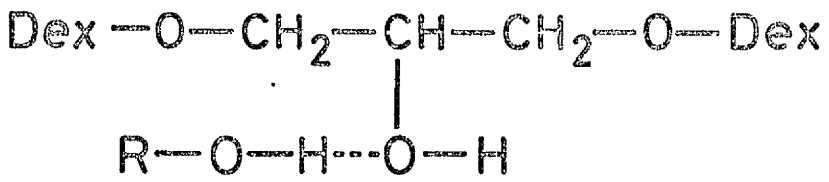
from a state of adsorption to one of partition in a gel system as continuous.[83] Electrostatic interactions of type (a) are observed in aqueous systems, especially when deionized water is the eluant, and will therefore not be important in the lipophilic gel systems under consideration. References 148 and 155 contain brief reviews of this topic.

Gel permeation has been described as a relatively weak separation method.[92] It gives rise to slight differences in the SEV of compounds of different molecular weight in straight-phase systems. Thus β-sitosterol, campesterol and cholesterol emerge in this order when benzene is used as mobile phase, and a homologous series of n-alcohols is eluted in order of decreasing molecular weight (see also ref. 92 for homologous series of n-alkanes, n-alkyl acetates and n-alkanols chromatographed on hydroxyalkyl LH-20 columns in benzene). High-resolution columns separating compounds only on a size basis have been constructed with polystyrene gels for use in polymer chemistry.[5,181,182] Very long columns may be required (up to 50 m) to achieve separations.[181] The extent to which these "size separations" are due to gel adsorption cannot easily be assessed, as the two modes of separation reinforce each other. A graph of log(elution volume) versus carbon number for the n-alcohols on the reversed-phase cholanyl gel is shown in Fig. 5.1. The curve is not a straight line (which would be expected from a purely partitioning chromatography system) suggesting that more than one mode is operating. Sjövall has made a similar observation on the separation of the isoprenologue vitamins $K_{2(10)}$ - $K_{2(40)}$ on a capillary column of methylated Sephadex.[83,183] Neither the graph of elution volume versus molecular weight[184] nor that of log(elution volume) versus

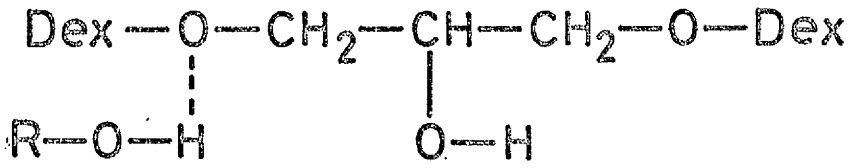
molecular weight is a straight line. Therefore the system is neither purely a gel permeation nor a partition system.

In the gel systems examined, peak widths have not shown the independence of elution volume that is a characteristic of GPC systems. An examination of Fig. 3.5 shows that the peak width increases as the SEV increases, and in this respect the system is similar to a partition system. All of the effects described have been observed for other lipophilic gels and it therefore appears general that gel permeation effects are of relatively minor importance in straight-phase or reversed-phase systems. When the polarity of the mobile phase is adjusted to equal that of the gel, a limit to the elution volume is observed in practice and separations dependent on molecular size can be obtained.[92]

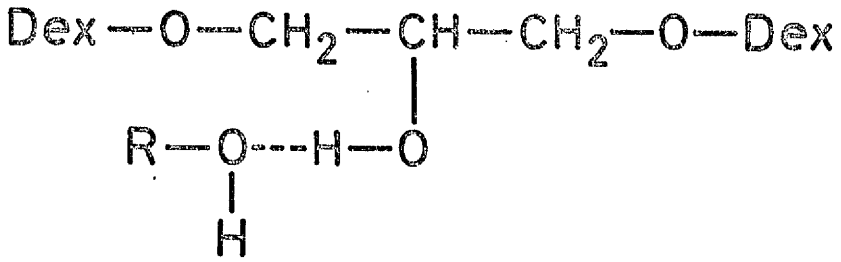
In Chapter 3, the symmetry of peaks and the independence of peak shape with respect to concentration were described for hydroxy-5β-cholanyl LH-20. This behaviour is normally indicative of linear concentration isotherms associated with partition systems. However, Determann has pointed out[136] that the polymer matrix of a swollen gel is readily accessible and effectively presents a very large surface area for adsorption of solutes. Linear isotherms may therefore be obtained for adsorption systems over an unusually wide concentration range. The solvent system used may be expected to be important in determining the mechanism of separation. If the eluant is a poor solvent for the sample being chromatographed, the stationary phase will tend to be favoured and partition forces will be dominant (for example, non-polar solutes have large elution volumes on columns eluted with methanol-heptane). If an eluant is used which is a good



(a)



(b)



(c)

Fig.5.2 Hydrogen-bonding schemes for the interaction of solutes containing hydroxyl functions with Sephadex G. (Reproduced from Ref.179).

solvent for the solute, then adsorption and permeation effects should be more prominent (as in the straight-phase benzene system).

Mention has already been made of the abnormal retention of aromatic substances on Sephadex G and LH-20 (para. 3.3). This effect has been ascribed to π-electron interactions between the solute and gel[185] and this idea has received some support in work by Streuli, who correlated π-electron resonance energy with adsorption.[157] However, aromatic compounds do not appear to be retained on fully lipophilic gels. The remaining types of adsorptive force are hydrogen bonding and hydrophobic bonding.

Hydrogen bonding has been implicated by numerous workers to explain the retardation of solutes containing hydroxylic, acidic or amino functions, beyond the elution volume their molecular size requires on columns of Sephadex G,[153-155,179] LH-20,[113,157] or hydroxyalkyl LH-20.[92] Present evidence suggests that the sites of bonding are the hydroxyether cross-links between the dextran chains or between the dextran and substituents[145,156] (Fig. 5.2). Brook and Munday have differentiated three types of hydrogen bond (Fig. 5.2(a)-(c)) according to the site and orientation of the two participating species.[179] The retardation of alcohols on straight-phase gel columns is almost certainly due to hydrogen bonding.

Morris and Morris[17] discuss hydrogen bonding in terms of donor and acceptor functions. Thus a water molecule can donate two hydrogen atoms and has one acceptor (oxygen) atom. Aliphatic alcohols such as methanol have one donor and one acceptor function. Ethers can act only as acceptors and some molecules containing non-accepting electronegative groups, for example chloroform, can act only as donors. It can

be seen from Fig. 5.2 that a hydroxylic solute has more opportunities to act as a donor than as an acceptor, because the ether linkages are acceptors only. Solvent systems containing species which do not act as donors or acceptors, or act as only one of these, give rise to straight-phase separations (for example benzene, chloroform or heptane). Elution with strong donors and acceptors, such as the lower alcohols or water, results in reversed-phase separations.

The separation of epimeric steroids on the straight-phase hydroxycyclohexyl column can be explained using a hydrogen bonding model to describe the solute-gel interaction. Equatorial hydroxyl groups in the steroid nucleus are usually less hindered than axial groups at the same position.[186] It is therefore reasonable to assume that equatorial alcohols will form hydrogen bonds more efficiently with the gel than the corresponding axial ones and the latter would be expected to be eluted first from the column. This is in agreement with experimental observations. An increased separation of epimeric hydroxylic steroids was observed on hydroxycholestanyl LH-20 compared to those obtained on hydroxycyclohexyl LH-20 and was ascribed to a stronger solute-gel interaction. The proximity of the bulky steroid substituent to the hydroxyether hydrogen bonding site must determine the steric requirements of those solutes that take part in hydrogen bonding.

The separation of 5α/Δ^5-steroids on hydroxycyclohexyl LH-20 may also be explained on the basis of a solute-gel interaction arising from hydrogen bonding. It was observed that, in the absence of a hydroxyl group at C-3 or on the formation of a group incapable of donating a hydrogen atom, using the terminology of Morris and Morris,[17]

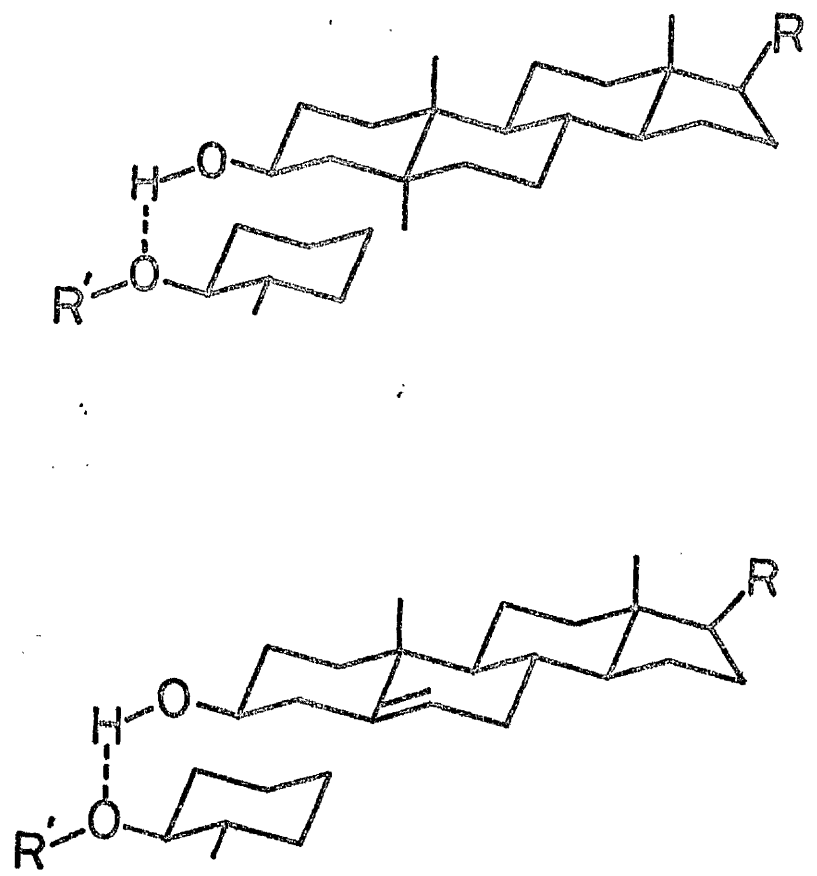


Fig.5.3 Interaction of the substituents of hydroxycyclohexyl LH-20 with 5α-cholestan-3β-ol and cholesterol.

no separation was achieved. A free hydroxyl group at C-3 is therefore necessary for the separation. One possible hydrogen bond complex between cholestanol and the hydroxycyclohexyl substituent is shown schematically in Fig. 5.3. It can be seen that the cyclohexyl ring can overlap with ring A of the steroid and with the A/B ring junction. Any change in the geometry at this point will influence the ability of the steroid to form a hydrogen bond because of steric limitations. For example, this column failed to separate hydroxylic steroids epimeric at C-3 when the configuration at C-5 was changed from α to β (with the exception of one pair of epimers). Removal of the hydrogen at C-5 will lower steric hindrance to hydrogen bond formation, and Δ^5-steroids are retained longer on the column than the corresponding 5α-steroids.

Hydrophobic interactions are the forces responsible for the association of non-polar groups in aqueous solutions.[187] Such association reduces the contact of the non-polar moieties with neighbouring water molecules and also tends to decrease the ordering of water molecules around the exposed non-polar solute. A decrease in the ordering of the water molecules results in an increase in entropy and therefore in a favourable free energy of formation of the hydrophobic bond. These forces play a role in maintaining the secondary and tertiary structure of proteins. Determann and Lampert have proposed[146] that hydrophobic bonds are responsible for the strong retention of lipophilic molecules observed during chromatography with columns of LH-20 in aqueous systems. In support of this hypothesis, it was shown empirically that the degree of retardation increased with temperature, which is in accord with an entropy-dependent bond. In polar solvents

other than water, also, non-polar groups may tend to contact each other, but this happens because the energy of solvent-solvent and solute-solute interaction is more favourable than solvent-solute interactions.[187] Such interactions have been described as "lyophobic" and are enthalpy dependent.[187] The bond is therefore weakened on increasing the temperature.

The effects of temperature on lipophilic gels were discussed in paragraph 3.2. The conclusion reached was that, in reversed-phase systems, elution volumes would decrease with temperature. Enthalpy-dependent forces would therefore be operating and not entropy-dependent forces analogous to the hydrophobic bond.

To return to the original question concerning the separation of enantiomers or phytosterol diastereoisomers, it seems likely that chiral lipophilic gels could be prepared containing polyfunctional substituents which would operate in the same manner as the optically active stationary phases described in Chapter 1. Adsorption of chiral solutes of suitable functionality (thereby leading to resolution) would be possible in straight-phase solvent systems. For non-polar enantiomers and phytosterols, it seems unlikely that the lyophobic interactions would ever be of sufficient specificity to permit their separation without increasing the column efficiency. The separation factors (α- values) of these compounds are extremely close to unity, and their separation will require columns containing high numbers of theoretical plates.

5.3 Areas of Further Work.

Several aspects requiring further investigation have already been mentioned. New gels can be prepared containing substituents of more

varied functionality. Polyfunctional, chiral, lipophilic gels might be prepared by attaching an amino acid residue to a lipophilic gel. The gels already prepared could be evaluated in other solvent systems with a wider range of compounds and with more elaborate chromatography techniques, in particular, recycle chromatography. Sjövall has suggested that "miscible" solvent systems containing several components may be more promising than eluants composed only of one or two solvents.[83] Some of the former type have already been tried (see for example ref. 90).

The effects of temperature on chromatography with these gels require more detailed examination. Information can be obtained thereby on the separation processes occurring in the gel. Also, if the temperature at which the chromatogram is performed were reduced, an enthalpy-dependent separation mode should be enhanced. At low temperatures, the interchange of conformations of phytosterol side chains might be reduced sufficiently to augment the slight differences in the distribution coefficients. It was noted earlier (Chapter 4) that 20α- and 20β-hydroxypregnanes could be separated whereas 24(R)- and 24(S)-hydroxycholesterol could not. This was ascribed to the lack of a strongly preferred conformation in the latter.

New modes of introduction of substituents will be needed in the future if complex reagents are to be used. The problems experienced with alicyclic oxides have already been discussed. Several base-catalysed substitution reactions were described in Chapter 2. On the whole, these were unsuccessful, and the product contained little or no substituent. One of the most likely methods of attaching groups may be through a silyl ether. The ease of formation of simple trimethylsilyl ethers is well known in the practice of GC. It would also be of

interest to analyse in detail the structure of the gels obtained by reaction with epoxides. One approach would be to synthesise substituted monomers, instead of a substituted polymer, by reaction of hydroxypropyl glucose with olefin oxides. Analysis using conventional spectroscopic techniques would then be possible.

Recent work by Almé and Nyström[188] and Almé, Sjövall and Bonsen[96] has introduced a new application of lipophilic gels as ion exchange resins. That this area of application is potentially large can be seen from the separations of phosphatidylcholines on the basis of the length and degree of unsaturation of the side-chains.[96]

Pharmacia, the manufacturers of Sephadex, now market gel materials for affinity chromatography[189] (CNBr-activated Sepharose 4B and Con A - Sepharose). This type of chromatography depends on the ability of many proteins and polypeptides to bind substrates specifically and reversibly. A gel containing a substituent which is specifically a substrate for a protein will retard this protein selectively in the course of the elution of a mixture of substances through the column. Much work in this field has been done by P. Cuatrecasas, who has recently reviewed the subject.[190,209]

One other field which holds scope for development is the preparation of gel stationary phases for use at high pressures. It is unlikely that Sephadex dextran will ever be suitable for this type of application. The use of an inert support (as in porous-layer beads) has been found to be unsuccessful.[191] Substitution of polystyrenes, which can be used at high pressures, has been carried out[192] but not much work has been done in the design of specific stationary phases. This is an important area of research, as much

of the current interest in liquid chromatography arises from the speed of separations which can be achieved. It is possible that future work will lead to chemically bonded stationary phases, with distinct chromatographic properties, which are based on inert, rigid supports. There are no gel phases available at present which are completely free from solute-gel interactions and which will give rise to separations on the basis of molecular size only. Porous glasses and silicas can be prepared with very precise pore widths and, when suitably treated to give inactive surfaces, may replace polymeric gels. Chemically bonded phases containing specific substituents could be prepared with presently available porous layer beads or microspheres. The segregation of chromatographic properties in this manner would facilitate the choice of stationary phase and enable separations to be carried out in a logical sequence.

All of the suggestions made in the foregoing paragraph appear feasible at present. The availability of so many potentially fruitful lines of research indicates that the growth of liquid chromatography will continue for several years to come.

SECTION II

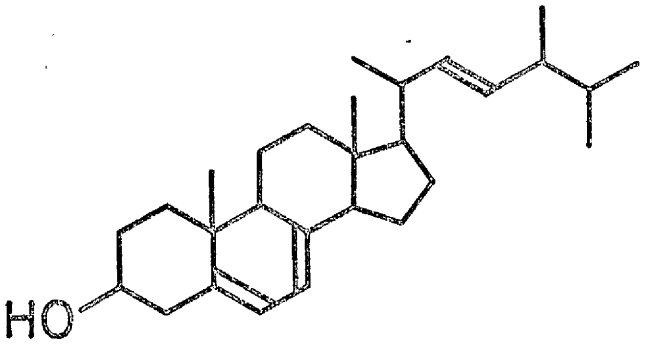
THE METABOLISM OF CHOLESTEROL BY PHYTOPHTHORA CACTORUM

SECTION II. THE METABOLISM OF CHOLESTEROL BY PHYTOPHTHORA CACTORUM.

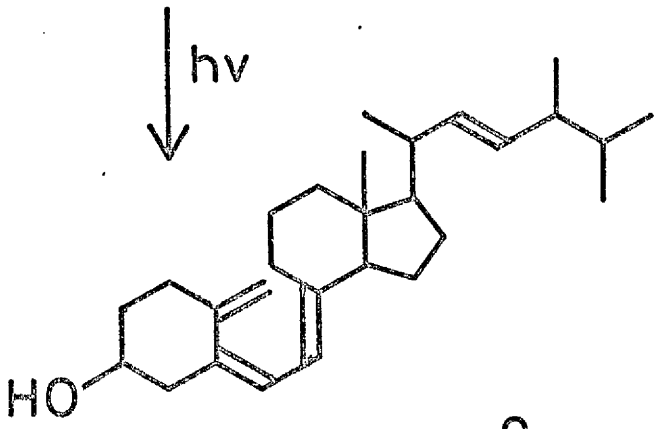
II.1 Introduction.

In the introduction to Section I, the role of steroids in the plant kingdom was discussed in general. This section is concerned with fungal steroids. Fungi are of considerable economic importance because of the damage that they cause to crops.[193] The use of fungicide sprays limits the effects of organisms such as Phytophthora infestans (the causal organism of potato blight) but repeated spraying is necessary. Because of the expense, fungicide sprays can be used successfully only in highly developed farming countries. From a different aspect, many fungi provide a useful and industrially important means of modifying steroids, for example by the introduction of hydroxyl functions to the steroid nucleus,[194] and industrial fermentations provide sources of ergosterol, the precursor for vitamin D_2.

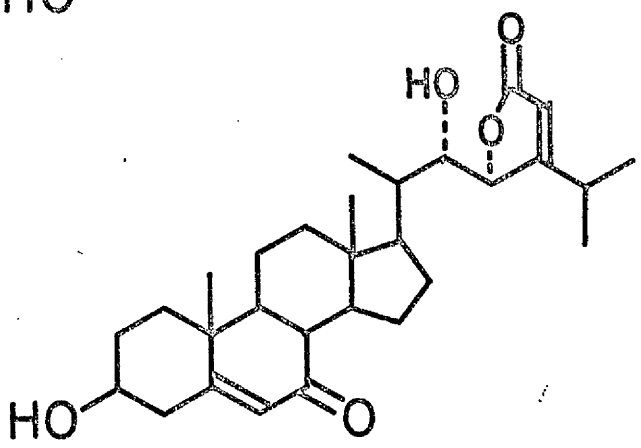
Many advantages are offered to the experimental biochemist and biologist as these organisms may be readily grown under reproducible conditions and in a comparatively short time compared to higher plants. Consequently, many studies of sterol biosynthesis have been carried out with fungi. The metabolic pathways have recently been reviewed.[195] As in animals, the initial product of squalene cyclisation in fungi is lanosterol, whereas cycloartenol is the first product formed in higher plants. Many subsequent modification reactions appear to exist, although, until the introduction of modern analytical methods, ergosterol was considered to be the only fungal sterol present in significant amounts, and few attempts were made to detect and characterise other steroids. A wide variety of steroids have now been



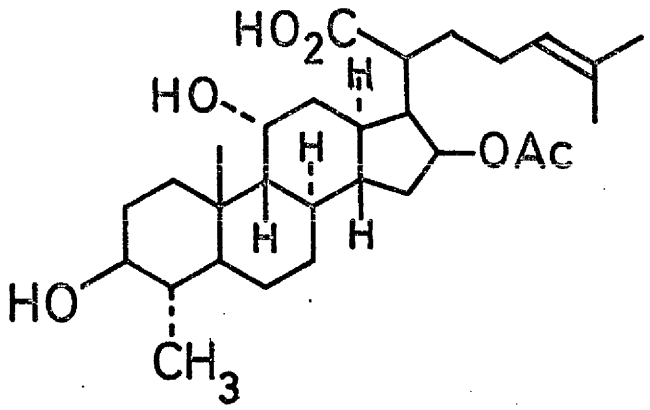
Ergosterol : sterol of yeast. Gives vitamin D_2 when irradiated.



Vitamin D_2 : the antirachitic factor.



Antheridiol : a fungal hormone produced by females of Achlya bisexualis.



× correct

Fusidic acid : a steroidal antibiotic from Fusidium coccineum.

Fig.II.1 Biologically important compounds associated with fungi.

identified in fungi,[28,195] and there appears to be much variation in the sterol composition of various species, although Weete does not consider it likely that the sterol pattern will serve as a useful chemotaxonomic character.[195]

Hendrix has reviewed the role of sterols in the growth and reproduction of fungi.[28] It may be recalled from Chapter 1 that steroids in plants were ascribed three possible functions as hormones, as membrane components and as precursors for other compounds. Similar functions appear to be fulfilled in the fungi as a whole, although all of these roles may not appear in any one species. The fungal hormone, antheridiol has been known for some time, and has comparatively recently been shown to be steroidal (see ref. 196 and references therein). Sterols have been shown to be present in fungal membranes[197] and appear to have a role in the uptake and metabolism of carbon sources in species of Mycoplasma.[198] Cholesterol decreases the release of sugar from phosphatidyl choline liposomes,[199] suggesting a role in membrane permeability. This idea is supported by the observation that polyene antibiotics interact with cellular sterols to disrupt the cellular permeability.[200]

A possible role of sterols in determining the disease resistance of Antirrhinum majus seedlings to attack by Pythium ultimum has been described earlier.[29] The sterol composition of potatoes has been related to the resistance to attack by Phytophthora infestans, a member of the Pythiaceae (comprising the genera Pythium and Phytophthora).[201] Steroid glycoalkaloids of potato peel have been shown to be toxic to some fungi[202] and similar compounds may account for the resistance of tomatoes to attack by nonpathogenic fungi.[203]

Fungi of the family Pythiaceae do not contain sterols, and, on media which are free from sterols, tend to grow vegetatively. However, when sterols are present, their rate of growth increases, sexual organs (oogonia and antheridia) appear, and oospores may be formed.[27] The reasons for the changes which occur in the presence of sterols are not known. The cell permeability may be affected or else the metabolic rate of the fungus may be changed. For example, Pythium ultimum takes up and catabolises glucose more rapidly in the presence of cholesterol than when this is absent.[204] Hendrix has analysed the metabolites of cholesterol in cultures of Pythium periplocum, grown under conditions suitable for sexual reproduction.[205] In the present work, the metabolites of cholesterol in Phytophthora cactorum were examined, as part of a larger project aimed at elucidating the role of sterols in the reproduction of these fungi.

II.2 Analysis of cholesterol metabolites.

A qualitative analysis only was carried out. Cultures of Phytophthora cactorum were grown by Dr. C.G. Elliott, of the Department of Botany, Glasgow University, in liquid medium in 100 ml conical flasks (stationary culture).[12] When the cultures were four days old, cholesterol containing 4-(^{14}C)-cholesterol was added in Tween 80 (40 mg sterol per litre of medium). After a further seven days, the mycelium was filtered, washed with water, and extracted in a Soxhlet thimble with acetone. Samples were removed for analytical TLC and the remainder of the extract was evaporated to give a yellow oil.

Analytical TLC was carried out using 5 x 20 cm plates (0.5 mm thickness). Two solvent systems were used: ethyl acetate-petroleum ether (60-80° BP fraction) in the proportions 5:95 v/v and 1:1 v/v

TABLE II.1 Analytical TLC of P. cactorum extract.

Solvent system	R_f value	Intensity of Autoradiograph	Colour with "ceric" spray	Comments
	0.00	strong	brown	More polar than cholesterol
	0.04	v. strong	red	Free cholesterol
	0.08	weak	not vis.	
5% EtOAc	0.16	weak	not vis.	
95% Petrol	0.28	weak	not vis.	
	0.53	weak	not vis.	
	0.56	weak	not vis.	
	0.61	v. strong	red	Cholesteryl esters
	0.00	weak	brown	
	0.07	weak		
	0.10	weak	pale brown where visible	
	0.24	weak		
	0.26	weak		
	0.63	strong	red	Cholesterol
	0.87	strong	brown	Cholesteryl esters

TABLE II.2 Silver nitrate TLC of P. cactorum lipids.

Material	No. of components by TLC	R_f Values	Comments
Cholesterol (standard)	1	0.01	
Cholesteryl oleate (standard)	1	0.49	
Cholesteryl palmitate (standard)	1	0.68	
Cholesteryl stearate (standard)	1	0.78	
Prep. TLC non-polar band	3	0.46	Cholesteryl oleate
		0.68	Cholesteryl palmitate
		0.79	Cholesteryl stearate
Prep. TLC polar band	1	0.0	Cholesterol

TABLE II.3 GLC of steryl esters directly on 1% SE-30.

Ester type	% of total ester fraction
Cholesterol + C_{14} acids	2.6
Cholesterol + C_{16} acids	12.7
Cholesterol + C_{18} acids	84.7

respectively. The plates were examined with the Panax radiochromatogram scanner, prior to visualisation with ceric ammonium sulphate and submission for autoradiography (for which service, thanks are due to Mr. N. Tait of the Botany Department). The plates were left in contact with photographic film for two weeks before development. The results of the TLC are summarised in Table II.1. R_f values for spots with associated radioactivity only are given. There were only two spots on these plates which were strongly radioactive. None of the materials having a lower R_f than cholesterol were detectable using the radiochromatogram scanner.

The remainder of the extract was applied to preparative TLC plates which were developed with ethyl acetate-petrol (5:95 v/v). The two strongly radioactive bands were located using the plate scanner. The silica was then scraped off and extracted with chloroform. Samples of each band were re-examined by analytical TLC on a 20 x 20 cm silica plate (0.25 mm thickness) containing 10% by weight of silver nitrate.[206] The following standards were also applied: cholesterol, cholesteryl oleate, cholesteryl palmitate and cholesterol stearate. The plate was developed in ethyl acetate-petrol (5:95 v/v) and examined in the same way as the first TLC plates. The results are summarised in Table II.2. It was seen that the non-polar band was a mixture of long-chain fatty acid esters of cholesterol and that the polar band was the free sterol. These results were confirmed by GLC analysis. The sterol esters were chromatographed directly on 1% SE-30 at 300°C (2 m column), and the sterol on 1% OV-1 at 225°C, both free and as the trimethylsilyl ether (Table II.3). The former column resolved the esters from C_{14}, C_{16} and C_{18} fatty acids but did not

TABLE II.4. GLC Analysis of Fatty Acid Methyl Esters.

Fatty Acid	A	B
C_{12}	0.70	
C_{14}	17.4	2.42
C_{16}	9.81	7.48
C_{16} (monounsaturated)	5.71	7.28
C_{18}	0.75	1.52
C_{18} (monounsaturated)	51.1	57.6
C_{18} (diunsaturated)	6.51	19.4
C_{18} (triunsaturated)	2.45	0.28
Unidentified long chain acids C_{20} or above	5.56	

Column A : % Fatty acid in triglyceride from fungus grown in the absence of cholesterol.

Column B : % Fatty acid in sterol esters.

resolve the esters according to the degree of unsaturation. Accordingly, the free fatty acids were obtained by alkaline hydrolysis (ethanolic KOH, 1 h at 40°C) and analysed by GLC on 10% EGSSX at 150° as the methyl esters. This stationary phase separated the methyl esters according to the degree of unsaturation of the chain as well as by carbon number (Table II.4).

For comparison purposes, the fatty acid pattern of the fungal triglyceride fraction was determined. The triglycerides were isolated by preparative TLC of an acetone extract of cultures of the fungus grown in the absence of sterol. The triglycerides were hydrolysed with ethanolic KOH and analysed as before (Table II.4). It can be seen that the fatty acid patterns from the sterol esters and from the triglycerides are similar. Oleic acid is the most abundant in both patterns. The main differences are in the relative amounts of C_{14} (myristic) acid, which is much lower in the sterol esters than in the triglycerides and C_{18} (linoleic acid: diunsaturated) which is higher in the esters than in the triglycerides. These may not be significant.

These results agree in part with those of Gain,[207] who carried out a similar analysis. The percentages of the fatty acids found in the sterol ester fraction are different (the principal acids being saturated). The conditions under which the fungus was grown may affect the fatty acid pattern. Gain also observed that the proportion of esters obtained was similar for both low and high cholesterol concentrations, whereas, in the conditions used in the present work, the proportion of esters increased as the cholesterol concentration increased. Hendrix observed that cholesterol was esterified in

cultures of Pythium periplocum.[205] However, significant amounts of a material more polar than cholesterol were also obtained. The structure has not yet been established, although it appears similar in its TLC character and stability to antheridiol. The small amounts of polar material obtained in the present work may be autoxidation products.

Recent work in this area by Dr. C.G. Elliott of the Department of Botany, Glasgow University, has shown that P. cactorum can differentiate between phytosterols diastereomeric at C-24. Thus dihydrobrassicasterol is less potent than campesterol as an agent for the induction of oogonia formation (the activity was measured on a statistical basis[27]). In view of the results discussed in Section I, it is unfortunate that this type of bioassay requires relatively large amounts of sterol, of the order of milligrams, and is therefore no more sensitive than NMR spectroscopy.

APPENDICES

APPENDIX 1. The LKB - SIMULATING GAS CHROMATOGRAPH.

Figs. A1.1 and A1.2 illustrate the chromatograph. The construction was begun by Dr. B.S. Middleditch, and wiring and other final connections were completed by the author. The components are:

(a) An LKB 9000 GLC oven. This accommodates columns of normal LKB design, length 10'. The oven temperature is controlled by (b). The LKB flash heater and Pye 104 flame ionisation detector (F.I.D.) are controlled by potentiometers mounted on the front panel. Thermocouples in the oven, FID and flash heater give readouts on the meter.

(b) A Perkin-Elmer Linear Temperature Programming Unit and precision oven control board.

(c) A Pye Ionisation Amplifier, as fitted to the 104 series gas chromatographs.

(d) A blower unit is mounted in the base of the instrument for forced ventillation of the cabinet during normal operation of the chromatograph.

(e) A thermal cutout device is mounted on the RHS side panel. An oven-mounted probe causes the oven power supply to be interrupted in the event of thermal runaway.

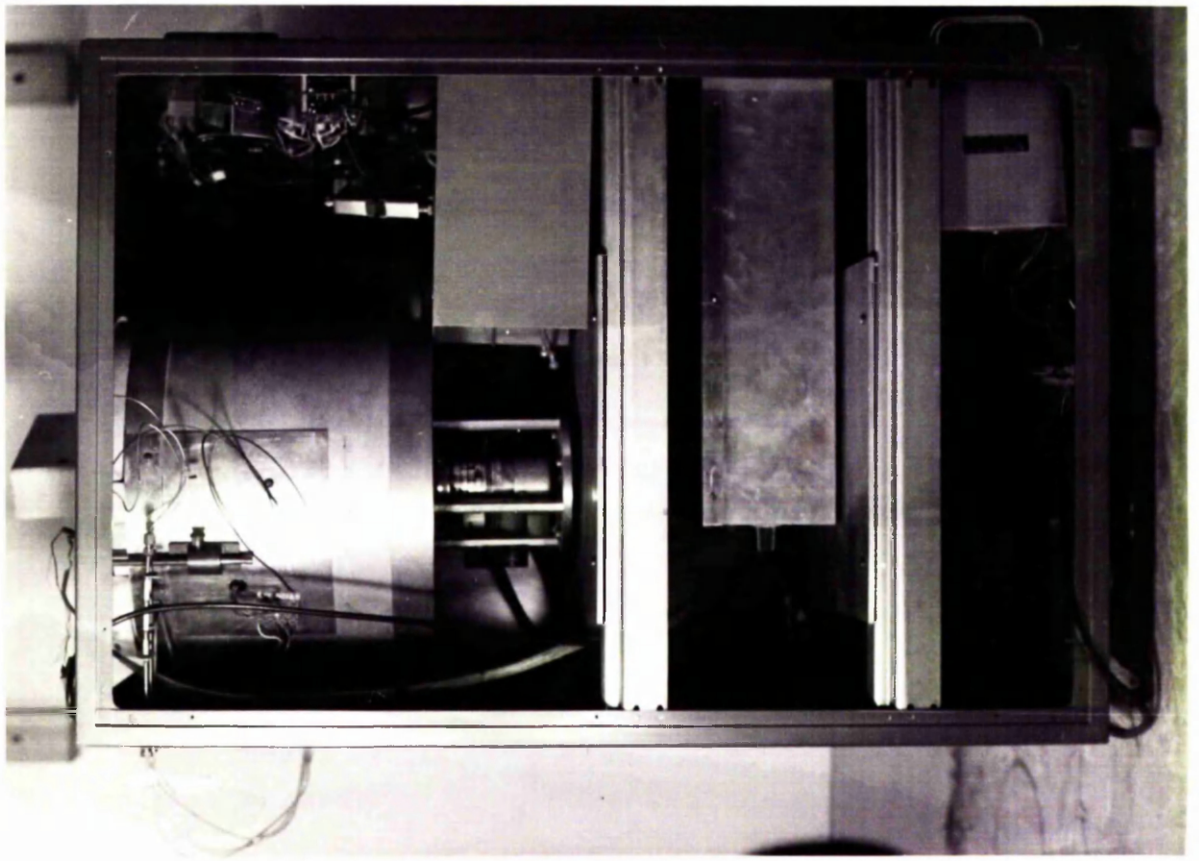
Fig.A1.1 The LKBS gas chromatograph, front view

Thermocouple readout, flash heater and detector heater potentiometers, and flow meter.

Perkin-Elmer Linear Temperature Programming Unit.

Pye Ionisation Amplifier.

Blower unit.



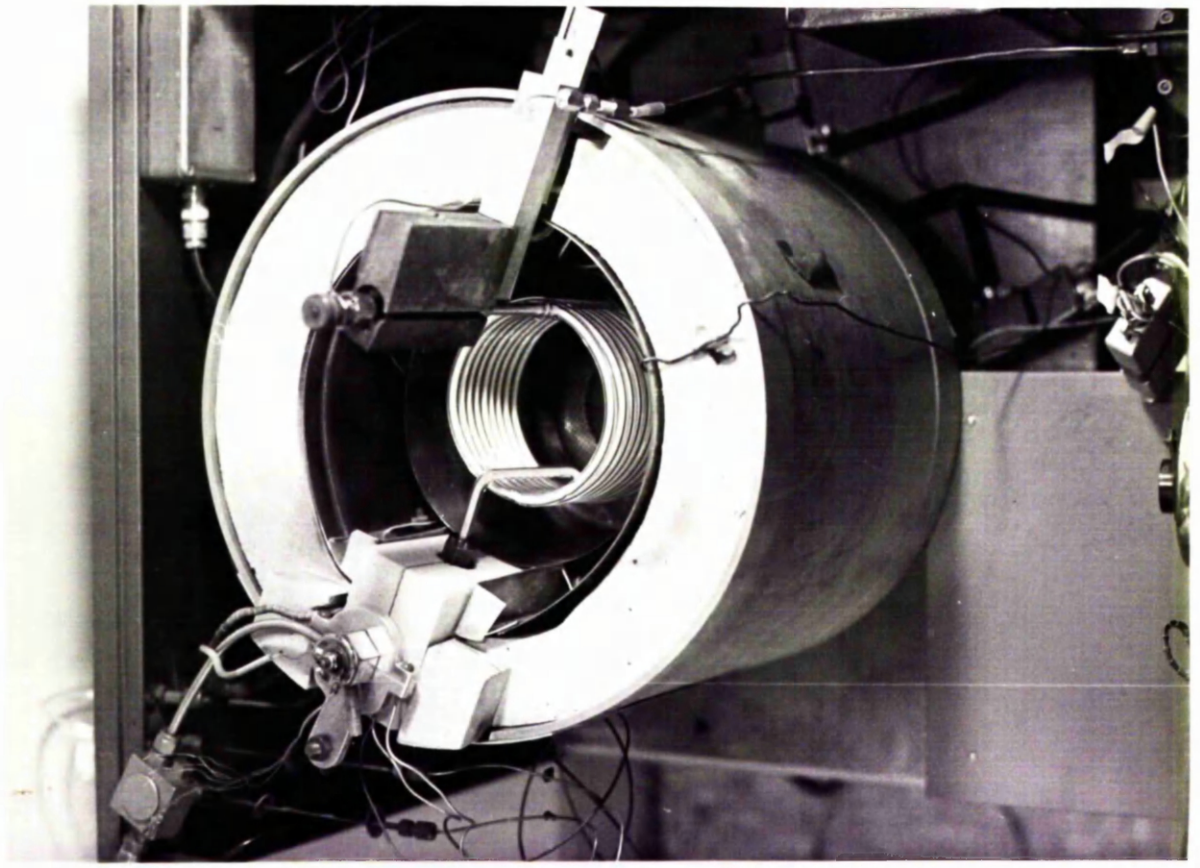


Fig.A1.2 The LKBS gas chromatograph, internal view of GC-oven and housing cabinet.

APPENDIX 2 : SPECTRA

Mass Spectra

A.2.1 23-Cholene

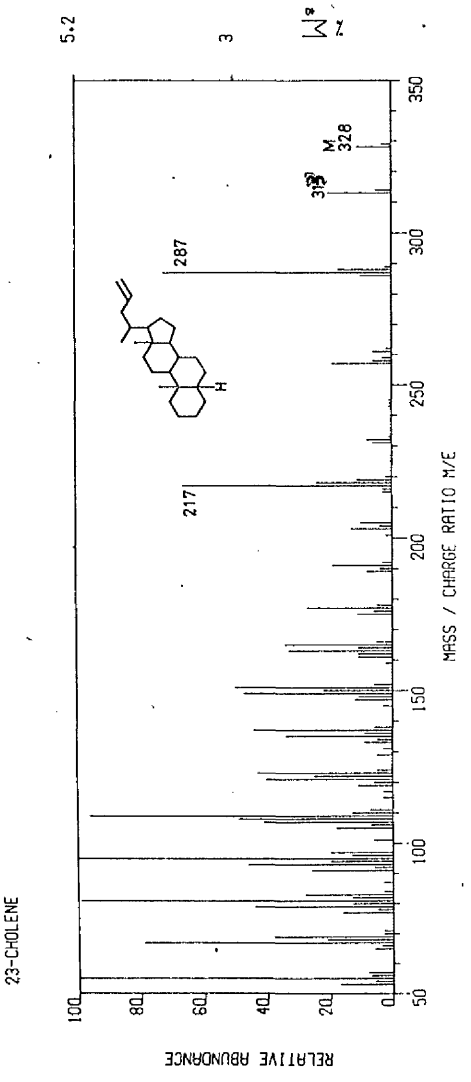
A.2.2 24-Butoxy-5β-cholane

A.2.3 5β-Cholan-23-al

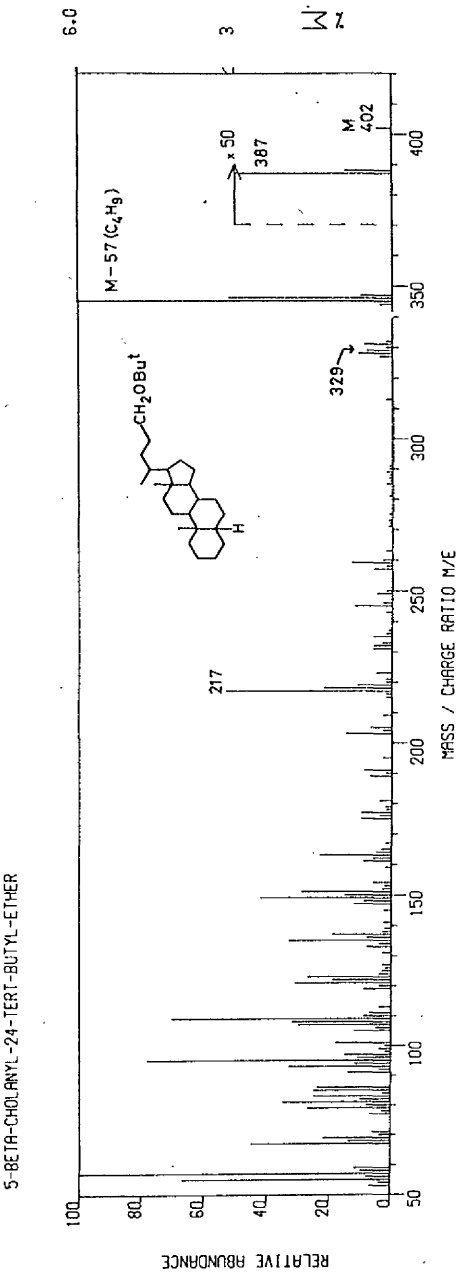
A.2.4 5β-Cholan-23-al O-methyl oxime

Liquid Chromatograph Trace

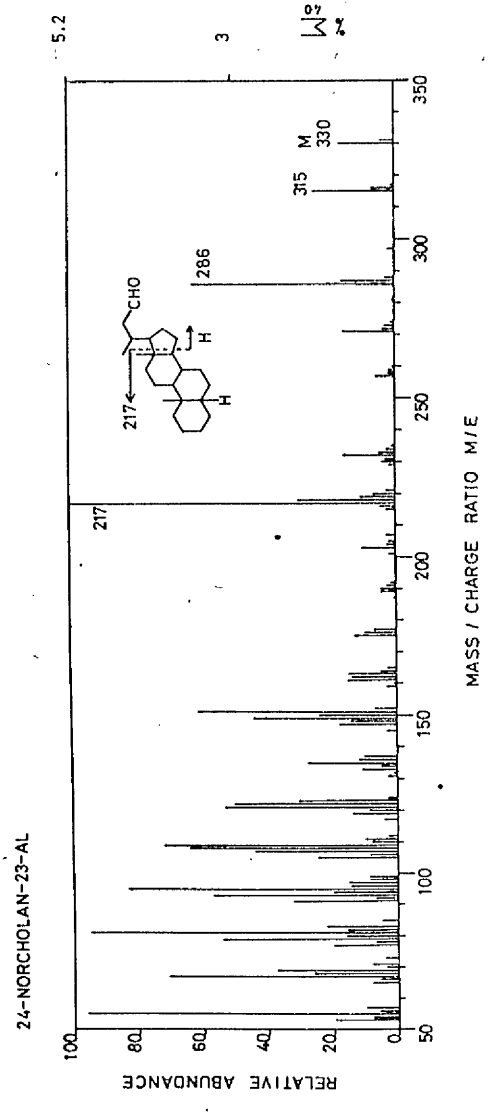
A.2.5 Separation of a mixture of hydroxylic steroid epimers on a straight-phase hydroxycyclohexyl LH-20 column eluted with benzene.


23-CHOLENE
RELATIVE ABUNDANCE
MASS / CHARGE RATIO M/E
M
328
287
217
5.2
3
%

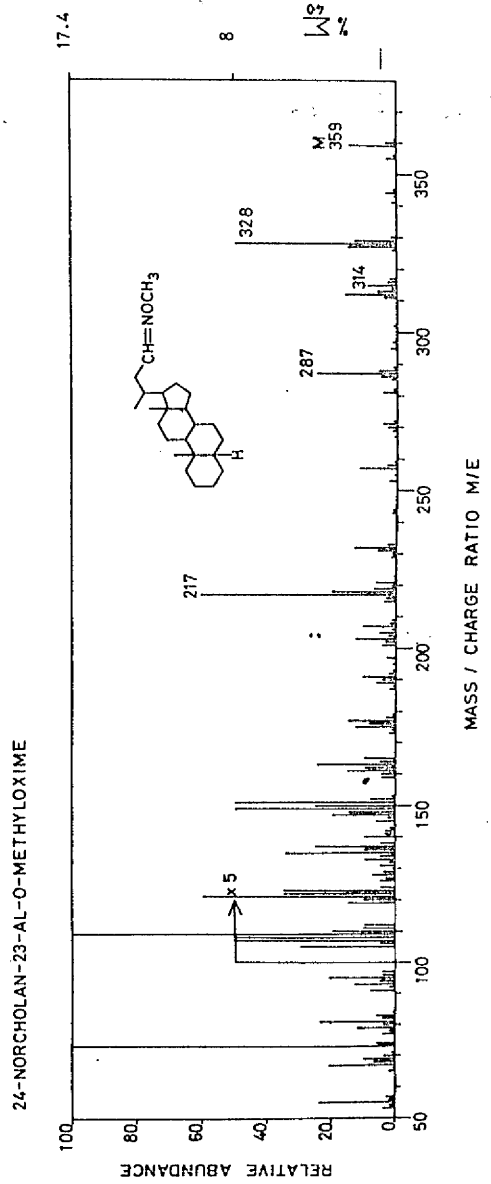
A.2.1


5-BETA-CHOLANYL-24-TERT-BUTYL-ETHER
RELATIVE ABUNDANCE
MASS / CHARGE RATIO M/E
M-57 (C4H9)
x 50
387
M
402
329
217
CH2OBut
6.0
3
%

A.2.2


24-NORCHOLAN-23-AL
5.2
3
M
330
315
286
217
CHO
H
H
217
RELATIVE ABUNDANCE
MASS / CHARGE RATIO M/E

A.2.3


24-NORCHOLAN-23-AL-O-METHYLOXIME
17.4
8
M
359
328
314
287
217
CH=NOCH3
H
x5
RELATIVE ABUNDANCE
MASS / CHARGE RATIO M/E

A.2.4

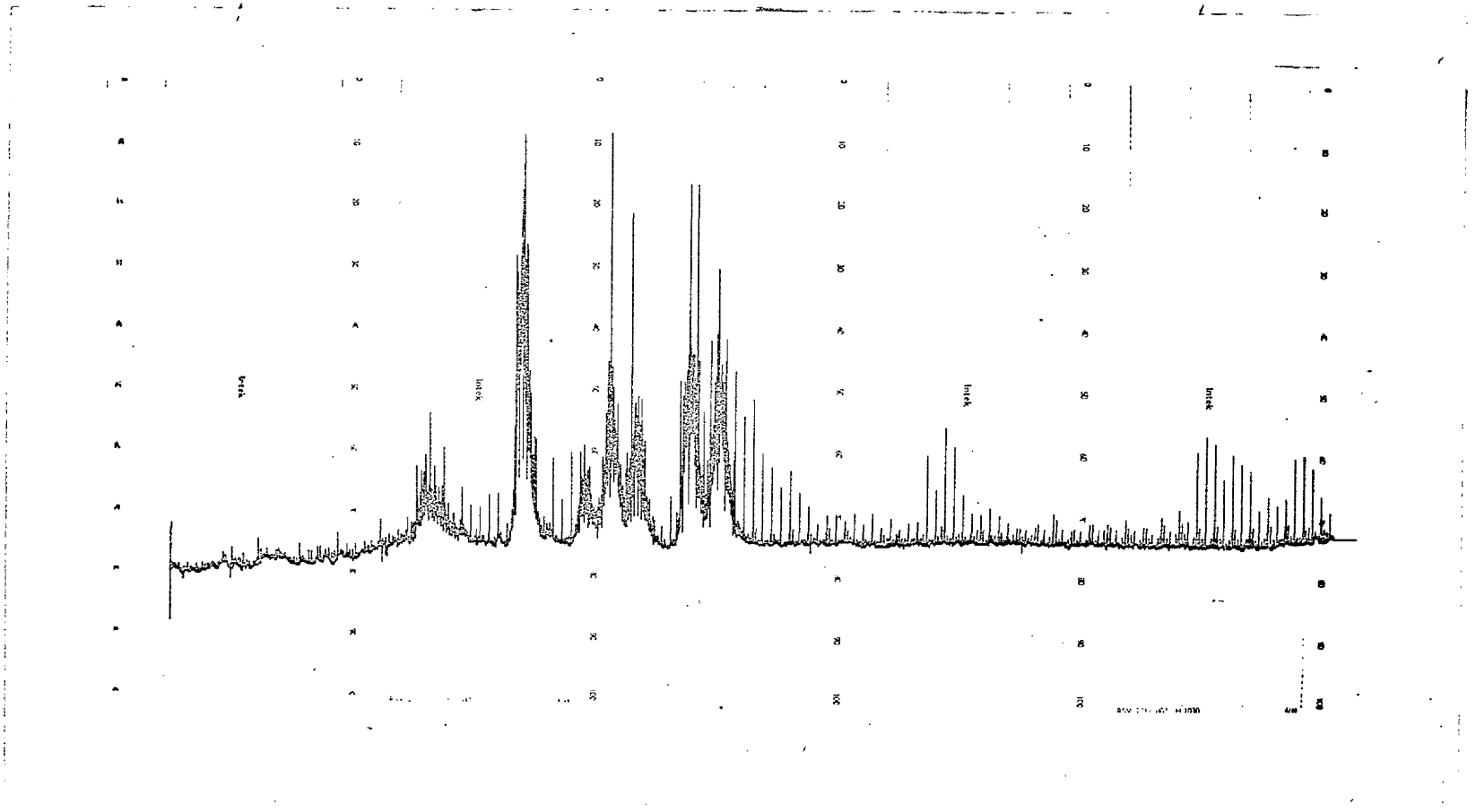


Fig.A.2.5 A reproduction of the original liquid chromatograph trace (obtained using the Sjovall-Haahti detector) used for the preparation of Fig 4.5. The noise level shown above is typical under normal operating conditions. The first peak on the left is an impurity and was omitted when the chart was traced for Fig.4.5.

APPENDIX 3 : TRIVIAL AND SYSTEMATIC NAMES OF STEROIDS

Trivial name	Systematic name
Androsterone	3α-Hydroxy-5α-androstan-17-one
Brassicasterol	24-(R)-Methyl-5,22-cholestadiene-3β-ol
Campesterol	24-(R)-Methyl-5-cholesten-3β-ol
Cholanic acid	5β-Cholan-24-oic acid
Cholanol	5β-Cholan-24-ol
Cholestane	5α-Cholestane
Cholestanol	5α-Cholestan-3β-ol
Cholestanone	5α-Cholestan-3-one
Cholesterol	5-Cholesten-3β-ol
Cholic acid	3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid
Coprostanol	5β-Cholestan-3β-ol
Dehydrocholic acid	3,7,12-Triketo-5β-cholan-24-oic acid
Dehydrodeoxycholic acid	3,12-Diketo-5β-cholan-24-oic acid
Dehydroepiandrosterone	3β-Hydroxy-5-androsten-17-one
Deoxycholic acid	3α,12α-Dihydroxy-5β-cholan-24-oic acid
Dihydrobrassicasterol	24-(S)-Methyl-5-cholesten-3β-ol
Epiandrosterone	3β-Hydroxy-5α-androstan-17-one
Epicholestanol	5α-Cholestan-3α-ol
Epicholesterol	5-Cholesten-3α-ol
Epicoprostanol	5β-Cholestan-3α-ol
Epietiocholanolone	3β-Hydroxy-5β-androstan-17-one
Etiocholanolone	3α-Hydroxy-5β-androstan-17-one
Estradiol	1,3,5(10)-Estratriene-3,17β-diol
Lanosterol	8,24-Lanostadiene-3β-ol
Lithocholic acid	3α-Hydroxy-5β-cholan-24-oic acid
Mestranol	17α-Ethynyl-1,3,5(10)-estratriene-3,17β-diol 3-methyl ether
Norethisterone	17α-Ethynyl-17β-hydroxy-4-estren-3-one
Nortestosterone	17β-Hydroxy-4-estren-3-one
Poriferasterol	24-(R)-Ethyl-5,22-cholestadiene-3β-ol
Progesterone	4-Pregnen-3,20-dione
β-Sitosterol	24-(R)-Ethyl-5-cholesten-3β-ol
Stigmasterol	24-(S)-Ethyl-5,22-cholestadiene-3β-ol
Testosterone	17β-Hydroxy-4-androsten-3-one

REFERENCES

1. L. Zechmeister in E. Heftmann (Ed.), Chromatography, Reinhold Publishing Corporation, New York, 1961, p. 3.

2. (a) A.J.P. Martin and R.L.M. Synge, Biochem. J., 1941, 35, 91.
   (b) Ident., Ibid., p. 1358.

3. R. Consden, A.H. Gordon and A.J.P. Martin, Biochem. J., 1944, 38, 224.

4. A.T. James and A.J.P. Martin, Biochem. J., 1952, 50, 679.

5. J.J. Kirkland (Ed.), Modern Practice of Liquid Chromatography, Wiley-Interscience, New York, 1971, (a) p. 49 (b) p. 375 (c) p. 436 (d) p. 65.

6. M.J.E. Golay in R.P.W. Scott (Ed.), Gas Chromatography, Butterworths, London, 1960, p. 139.

7. J. Bohemen and J.H. Purnell, J. Chem. Soc., 1961, 360.

8. I. Halász and C. Horváth, Anal. Chem., 1964, 36, 1178.

9. J.J. Kirkland and J.J. DeStefano, J. Chromatog. Sci., 1970, 8, 309, and references therein.

10. J.J. Kirkland, J. Chromatog. Sci., 1972, 10, 593.

11. I. Halász and I. Sebestian, Angew. Chem. I.E., 1969, 8, 453.

12. (a) M. Novotny, S.L. Bektesh, K.B. Denson, K. Grohmann and W. Parr, Anal. Chem., 1973, 45, 971.
    (b) W.A. Aue and C.R. Hastings, J. Chromatogr., 1969, 42, 319.

13. (a) W.A. Pavelich and R.A. Livigni, J. Polym. Sci., Part C, 1967, 215 (Pub. 1968).
    (b) D.J. Harmon, J. Appl. Polym. Sci., 1967, 11, 1333.
    (c) J.G. Hendrickson, Ibid., 1967, 11, 1419.

14. C.D. Scott, Advan. Clin. Chem., 1972, 15, 1.

15. C.A. Burtis, J. Chromatogr., 1970, 52, 97.

16. I.E. Bush, The Chromatography of Steroids, Pergamon Press, Oxford, 1961.

17. C.J.O.R. Morris and P. Morris, Separation Methods in Biochemistry, Sir Isaac Pitman and Sons Ltd., London, 1963, p. 438.

18. (a) S. Siggia and R.A. Dishman, Anal. Chem., 1970, 42, 1223.
    (b) F.A. Fitzpatrick, S. Siggia and J. Dingman, Sr., Ibid., 1972, 44, 2211.

19. R.A. Henry, J.A. Schmit and J.F. Dieckman, J. Chromatog. Sci., 1971, 9, 513.

20. B.L. Karger and L.V. Berry, Clin. Chem., 1971, 17, 757.

21. E. Heftmann, Steroid Biochemistry, Academic Press, London, 1970.

22. E. Heftmann, Lipids, 1971, 6, 128.

23. C. Grunwald, Plant Phys., 1968, 43, 484.

24. T.W. Goodwin in J.B. Pridham (Ed.), Terpenoids in Plants, Academic Press, 1967, p. 1.

25. J. Kopcewicz, Phytochem., 1971, 10, 1423.

26. A.M. Gawienowski, R.W. Cheney, Jr. and H.V. Marsh, Phytochem., 1971, 10, 2033.

27. C.G. Elliott, M.R. Hendrie and B.A. Knights, J. Gen. Microbiol., 1966, 42, 425.

28. J.W. Hendrix, Ann. Rev. Phytopathol., 1970, 8, 111.

29. H.M. Mellano, D.E. Munnecke and J.J. Sims, Phytopathol., 1970, 60, 943.

30. (a) E. Lederer, Quart. Revs., 1969, 23, 453.
(b) T.W. Goodwin, Biochem. J., 1971, 123, 293.
(c) L.J. Goad, F.F. Knapp, J.R. Lenton and T.W. Goodwin, Biochem. J., 1972, 129, 219.

31. B.A. Knights and A.M.M. Berrie, Phytochem., 1971, 10, 131.

32. B.A. Knights, Chem. in Brit., 1973, 9, 106, and references therein.

33. L.M. Bolger, H.H. Rees, E.L. Ghisalberti, L.J. Goad and T.W. Goodwin, Biochem. J., 1970, 118, 197.

34. M.J. Thompson, S.R. Dutchy, G.W. Patterson and E.L. Golden, Phytochem., 1972, 11, 1781.

35. D.S. Ingram, B.A. Knights, I.J. McEvoy and P. McKay, Phytochem., 1968, 7, 1241.

36. C.W. Shoppee, Chemistry of the Steroids, Butterworths, London, 1964.

37. A. Windaus and A. Welsch, Chem. Ber., 1909. 42, 612.

38. E. Fernholz and H.E. Stavely, J. Am. Chem. Soc., 1940, 62, 428, 1875.

39. E. Fernholz and H.B. MacPhillamy, J. Am. Chem. Soc., 1941, 63, 1155.

40. P.H. Boyle, Quart. Revs., 1971, 25, 323.

41. S.H. Wilen, Topics in Stereochem., 1971, 6, 107.

42. G. Losse and K. Kuntze, Z. Chem., 1970, 10, 22.

43. C.J.W. Brooks, M.T. Gilbert and J.D. Gilbert, Anal. Chem., 1973, 45, 896.

44. M.W. Anders and M.J. Cooper, Anal. Chem., 1971, 43, 1093.

45. (a) B. Halpern and J.W. Westley, Biochem. Biophys. Res. Commun., 1965, 19, 361.
(b) G.E. Pollock, V.I. Oyama and R.D. Johnson, J. Gas Chromatog., 1965, 3, 174.

46. W. Pereira, V.A. Bacon, W. Patton, B. Halpern and G.E. Pollock, Anal. Lett., 1970, 3, 23.

47. B. Feibush, Anal. Chem., 1971, 43, 1098.

48. M. Sanz-Burata, J. Inurre-Perez and S. Julia-Arechaga, Chem. Abs., 1971, 75, 5090.

49. M. Hamberg, Chem. Phys. Lipids, 1971, 6, 152.

50. S. Hammarström and M. Hamberg, Anal. Biochem., 1973, 52, 169.

51. A.H. Beckett and B. Testa, J. Chromatogr., 1972, 69, 285.

52. F. Raulin and B.N. Khare, J. Chromatogr., 1973, 75, 13.

53. G.E. Pollock, Anal. Chem., 1972, 44, 2368.

54. J.M. Cross, B.F. Putney and J. Bernstein, J. Chromatog. Sci., 1970, 8, 679.

55. E. Romann, A.J. Frey, P.A. Stadler and A. Eschenmoser, Helv. Chim. Acta, 1957, 40, 1900.

56. H. Gerlach, Helv. Chim. Acta, 1968, 51, 1587.

57. M. Koreeda, G. Weiss and K. Nakanishi, J. Am. Chem. Soc., 1973, 95, 239.

58. E.L. Eliel, Stereochemistry of Carbon Compounds, McGraw-Hill Book Company Inc., New York, 1962, p. 61, and references therein.

59. R. Curti and U. Colombo, J. Am. Chem. Soc., 1952, 74, 3961.

60. A.G. Ogston, Nature, 1948, 162, 963.

61. S. Nakaparksin, P. Birell, E. Gil-Av and J. Oró, J. Chromatog. Sci., 1970, 8, 177.

62. E. Bayer, E. Gil-Av, W.A. König, S. Nakaparksin, J. Oró and W. Parr, J. Am. Chem. Soc., 1970, 92, 1738.

63. J.A. Corbin and L.B. Rogers, Anal. Chem., 1970, 42, 1786.

64. W. Parr and P.Y. Howard, J. Chromatogr., 1972, 67, 227.

65. B. Feibush, E. Gil-Av and T. Tamari, J. Chem. Soc. (Perkin Trans. II), 1972, 1197.

66. R.E. Leitch, H.L. Rothbart and W. Rieman III, J. Chromatogr., 1967, 28, 132.

67. G. Manecke and W. Lamer, Angew. Makromol. Chem., 1973, 24, 57.

68. R.J. Baczuk, G.K. Landram, R.J. Dubois and H.C. Dehm, J. Chromatogr., 1971, 60, 351.

69. J.H. Purnell, J. Chem. Soc., 1960, 1268.

70. K.J. Bombaugh, W.A. Dark and R.F. Levangie, Separation Sci., 1968, 3, 375.

71. J.C. Giddings, Anal. Chem., 1967, 39, 1027.

72. D.C. Locke, Advan. Chromatogr., 1969, 8, 47.

73. J.C. Giddings and R.A. Keller in E. Heftmann (Ed.), Chromatography, Reinhold Publishing Corporation, New York, 1961, p. 92.

74. A.J.P. Martin, Ann. Rev. Biochem., 1950, 19, 517.

75. D.M.W. Anderson, I.C.M. Dea and A. Hendrie, Talanta, 1971, 18, 365.

76. J. Boldingh, Rec. Trav. Chim., 1950, 69, 247.

77. F.J. Ritter and J. Hartel, J. Chromatogr., 1958, 1, 461.

78. D. Kritchevsky and M.R. Kirk, J. Am. Chem. Soc., 1952, 74, 4484.

79. J. Hirsch, J. Lipid Res., 1963, 4, 1.

80. T. Seki, J. Chromatogr., 1959, 2, 667.

81. H.P. Gregor, G.K. Hoeschele, J. Potenza, A.G. Tsuk, R. Feinland, M. Shida and P. Teyssie, J. Am. Chem. Soc., 1965, 87, 5525.

82. P.I. Brewer, Nature, 1961, 190, 625.

83. J. Sjövall, E. Nyström and E. Haahti, Advan. Chromatogr., 1968, 6, 119.

84. Sephadex-Gel Filtration in Theory and Practice, Pharmacia Fine Chemicals, Uppsala, Sweden, 1966.

85. H. Determann, Angew. Chem. I.E., 1964, 3, 608.

86. W. Heitz, H. Ullner and H. Höcker, Makromol. Chem., 1966, 98, 42.

87. E. Nyström, Chromatography on Lipophilic Derivatives of Sephadex, Dissertation, Department of Chemistry, Karolinska Institutet, Stockholm, 1968.

88. Sephadex LH-20, for Gel Filtration in Organic Solvents, Pharmacia Fine Chemicals, Uppsala, Sweden, 1967.

89. J. Ellingboe, E. Nyström and J. Sjövall, Biochim. Biophys Acta, 1968, 152, 803.

90. J. Ellingboe, E. Nyström and J. Sjövall, J. Lipid Res., 1970, 11, 266.

91. R.A.B. Keates and C.J.W. Brooks, Biochem. J., 1969, 114, 16P.

92. C.J.W. Brooks and R.A.B. Keates, J. Chromatogr., 1969, 44, 509.

93. E. Nyström and J. Sjövall, Anal. Letts., 1973, 6, 155.

94. H. Tanaka and K. Konishi, J. Chromatogr., 1972, 64, 61.

95. J. Ellingboe, B. Almé and J. Sjövall, Acta Chem. Scand., 1970, 24, 463.

96. B. Almé, J. Sjövall and P.P.M. Bonsen, Anal. Letts., 1971, 4, 695.

97. P.M. Hyde and W.H. Elliott, J. Chromatogr., 1972, 67, 170.

98. K. Morikawa and Y. Hirose, Tetrahedron Letts., 1969, 1799.

99. A. Aebi and T. Waaler, Uber die Inhaltsstoffe von Petasites hybridus (L) Fl. Wett., Verlag Helbing und Lichtenhahn, Basel, 1959.

100. R.A.B. Keates, Ph.D. Thesis, Chemistry Department, University of Glasgow, 1970.

101. F.G. Mann and B.C. Saunders, Practical Organic Chemistry, 4th Edition, Longmans, London, 1960.

102. E.C. Horning, W.J.A. VandenHeuvel and B.G. Creech, Methods of Biochemical Analysis, 1963, 11, 69.

103. D.D. Perrin, W.L.F. Armarego and D.R. Perrin, Purification of Laboratory Chemicals, 1st Edition, Pergamon Press, Oxford, 1966.

104. H. Hartley and H.R. Raikes, J. Chem. Soc., 1925, 127, 524.

105. J.A. Moore and D.E. Reed, Org. Synth., 1961, 41, 16.

106. Personal communication by E.J. Eisenbraun, Oklahoma State Univ., to L.F. Fieser; see Ref. 113, Vol. II.

107. B. Riegel, R.B. Moffett and A.V. McIntosh, Org. Synth., 1944, 24, 41.

108. K. Tsuda, S. Nozoe and K. Ohata, Chem. Pharm. Bull., 1963, 11, 1265.

109. Huang-Minlon, J. Am. Chem. Soc., 1949, 71, 3301.

110. F. Wessely and W. Swoboda, Monatsh. Chem., 1951, 82, 437.

111. N.F. Wood and F.C. Chang, J. Org. Chem., 1965, 30, 2054.

112. S.G. Wyllie and C. Djerassi, J. Org. Chem., 1968, 33, 305.

113. L.F. Fieser and M. Fieser, Reagents for Organic Synthesis, Vols. 1-3, Wiley, New York, 1967, 1969 and 1972.

114. E.B. Hershberg, E. Oliveto, M. Rubin, H. Staedule and L. Kuhlen, J. Am. Chem. Soc., 1951, 73, 1144.

115. W.F. Bruce, Org. Synth. Coll. Vol. II, John Wiley and Sons Inc., New York, 1943.

116. J.E. Herz, E. González and B. Mandel, Aust. J. Chem., 1970, 23, 857.

117. K.L. Williamson and W. S. Johnson, J. Org. Chem., 1961, 26, 4563.

118. M. Hanack and R. Hähnle, Chem. Ber., 1962, 95, 191.

119. L. Borowiecki and Y. Chrétien-Bessière, Bull. Soc. Chim. Fr., 1967, 2364.

120. L.M. Jackman and S. Sternhell, Applications of NMR Spectroscopy in Organic Chemistry, 2nd Edition, Pergamon Press, Oxford, 1969, p. 84.

121. J.C. Giddings and K.L. Mallik, Anal. Chem., 1966, 38, 997.

122. P.B. Hamilton, Anal. Chem., 1958, 30, 914.

123. P.B. Hamilton, Anal. Chem., 1960, 32, 1779.

124. P.B. Hamilton, D.C. Bogue and R.A. Anderson, Anal. Chem., 1960, 32, 1782.

125. L. Fischer, An Introduction to Gel Chromatography, North Holland Publishing Co., Amsterdam and London, 1969, p. 228.

126. J. Ellingboe, E. Nyström and J. Sjövall, in J.M. Lowenstein and R.B. Clayton (Eds.), Methods in Enzymology, Academic Press Inc., New York, 1968.

127. K.W. Pepper, D. Reichenberg and D.K. Hale, J. Chem. Soc., 1952, 3129.

128. F. Helfferich, Ion Exchange, McGraw-Hill, New York, 1962, p. 230.

129. J.J. Kirkland, J. Chromatog. Sci., 1969, 7, 7.

130. J.J. Kirkland, J. Chromatog. Sci., 1969, 7, 361.

131. J. Kwok, L.R. Snyder and J.C. Sternberg, Anal. Chem., 1968, 40, 118.

132. C.D. Scott, W.F. Johnston and V.E. Walker, Anal. Biochem., 1969, 32, 182.

133. E. Haahti, T. Nikkari and J. Kärkkäinen, J. Gas Chromatog., 1966, 4, 12.

134. J.M. Goodson and V. DiStefano, J. Chromatogr., 1969, 45, 139.

135. C.G. Scott and P. Bommer, in A. Zlatkis (Ed.), Advan. Chromatogr., Proc. Int. Symp., 6th 1970, p. 416.

136. H. Determann, Gel Chromatography, Springer-Verlag, Berlin, 1968.

137. E. Nyström and J. Sjövall, Anal. Biochem., 1965, 12, 235.

138. E. Nyström, Arkiv Kemi, 1968, 29, 99.

139. B. Coxon, Advan. Carbohyd. Chem. Biochem., 1972, 27, 7.

140. R.J. Gritter in S. Patai (Ed.), The Chemistry of the Ether Linkage, Interscience Publishers, London, 1967, p. 373.

141. A. Rosowski in A. Weissberger (Ed.), Heterocyclic Compounds with Three and Four-Membered Rings, Interscience Publishers, New York, 1964, p. 1.

142. D.N. Kirk, Chem. in Ind., 1973, 109.

143. J.C. Buchanan and H.Z. Sable, in B.S. Thyagarajan (Ed.), Selective Organic Transformations, Vol. 2, Wiley-Interscience, New York, 1972, p. 1.

144. H.C. Chitwood and B.T. Freure, J. Am. Chem. Soc., 1946, 68, 680.

145. K. Lampert and H. Determann, J. Chromatogr., 1971, 56, 140.

146. H. Determann and K. Lampert, J. Chromatogr., 1972, 69, 123.

147. T.H. Holmdahl and J. Sjövall, Steroids, 1971, 18, 69.

148. K.W. Williams, Lab. Practice, 1972, 21, 667.

149. G.T. Stevenson, J. Chromatogr., 1968, 37, 116.

150. W.H.S. George, Nature, 1962, 195, 155.

151. R.A. Anderson, unpublished data.

152. M. Joustra, B. Söderqvist and L. Fischer, J. Chromatogr., 1967, 28, 21.

153. J. Porath, Biochim. Biophys. Acta, 1960, 39, 193.

154. B. Gelotte, J. Chromatogr., 1960, 3, 330.

155. J.-C. Janson, J. Chromatogr., 1967, 28, 12.

156. H. Determann and I. Walter, Nature, 1968, 219, 604.

157. C.A. Streuli, J. Chromatogr., 1971, 56, 219.

158. H.H. Oelert, Z. Anal. Chem., 1969, 244, 91.

159. M. Wilk, J. Rochlitz and H. Bende, J. Chromatogr., 1966, 24, 414.

160. E. Makowetz, K. Mueller and H. Spitzy, Microchem. J., 1966, 10, 194.

161. Dictionary of Organic Compounds, Vol. 5, 4th Edition, Eyre and Spottiswoode Publishers Ltd., E. and F.N. Spon Ltd., London, 1965, p. 3232.

162. J.D. Gilbert, personal communication.

163. E.B. Kyba, K. Koga, L.R. Sousa, M.G. Siegel and D.J. Cram, J. Am. Chem. Soc., 1973, 95, 2692.

164. R.C. Helgeson, K. Koga, J.M. Timko and D.J. Cram, J. Am. Chem. Soc., 1973, 95, 3021.

165. R.C. Helgeson, J.M. Timko and D.J. Cram, J. Am. Chem. Soc., 1973, 95, 3023.

166. N.S. Bowman, G.T. McCloud and G.K. Schweitzer, J. Am. Chem. Soc., 1968, 90, 3848.

167. R.H. Shapiro and M.J. Heath, J. Am. Chem. Soc., 1967, 89, 5734.

168. G. Kaufman, F. Cook, H. Shechter, J. Bayless and L. Friedman, J. Am. Chem. Soc., 1967, 89, 5736.

169. W.G. Dauben, M.E. Lorber, N.D. Vietmeyer, R.H. Shapiro, J.H. Duncan and K. Tomer, J. Am. Chem. Soc., 1968, 90, 4762.

170. G.M.L. Cragg, C.W. Davey, D.N. Hall, G.D. Meakins, E.E. Richards and T.L. Whateley, J. Chem. Soc. (C)., 1966, 1266.

171. G.M. Cameron and J.G. Marsden, Chem. in Brit., 1972, 8, 381.

172. H.O. House, J. Am. Chem. Soc., 1955, 77, 5083.

173. M. Tiffeneau and B. Tchoubar, Compt. Rend., 1941, 212, 581.

174. G.R. Clemo and J. Ormston, J. Chem. Soc., 1933, 362.

175. B. Tchoubar, Compt. Rend., 1942, 214, 117.

176. T.A. Crabb and K. Schofield, J. Chem. Soc., 1958, 4276.

177. R. Huisgen, E. Rauenbusch and G. Seidl, Chem. Ber., 1957, 90, 1958.

178. P. Eneroth and E. Nyström, Biochim. Biophys. Acta, 1967, 144, 149.

179. A.J.W. Brook and K.C. Munday, J. Chromatogr., 1970, 47, 1.

180. L.F. Martin, N.R. Bertonière and S.P. Rowland, J. Chromatogr., 1972, 64, 263.

181. K.J. Bombaugh, W.A. Dark and R.F. Levangie, J. Chromatog. Sci., 1969, 7, 42.

182. W. Heitz, B. Bömer and H. Ullner, Makromol. Chem., 1969, 121, 102.

183. E. Nyström and J. Sjövall, J. Chromatogr., 1966, 24, 212.

184. J.R. Whitaker, Anal. Chem., 1963, 35, 1950.

185. D. Eaker and J. Porath, Separ. Sci., 1967, 2, 507.

186. L.F. Fieser and M. Fieser, Steroids, Reinhold Publishing Co., New York, 1967.

187. G. Némethy, Angew. Chem. I.E., 1967, 6, 195.

188. B. Almé and E. Nyström, J. Chromatogr., 1971, 59, 45.

189. CNBr-Activated Sepharose 4B and Con A-Sepharose, Pharmacia Fine Chemicals AB, Box 604, Uppsala, Sweden, 1972.

190. P. Cuatrecasas, in G.R. Stark (Ed.), Biochemical Aspects of Reactions on Solid Supports, Academic Press, New York, 1971, p. 71.

191. N.I. Olsson (Pharmacia AB, Uppsala.), personal communication.

192. S.B. Makarova and E.V. Egorov, Chem. Abs., 1972, 77, 75861e.

193. D. Woodcock, Chem. in Brit., 1971, 7, 415.

194. W. Charney and H.L. Herzog, Microbiological Transformations of Steroids, Academic Press, New York, 1967.

195. J.D. Weete, Phytochem. 1973, 12, 1843.

196. A.W. Barksdale, Science, 1969, 166, 831.

197. J.H. Sietsma and R.H. Haskins, Can. J. Microbiol., 1967, 13, 361.

198. P.F. Smith, Abh. Deut. Akad. Wiss. Berlin , Kl. Med., 1969, 1968, 813.

199. J. De Grier, J.G. Mandersloot and L.L.M. Van Deenen, Biochim. Biophys. Acta, 1969, 173, 143.

200. D. Gottlieb and P.D. Shaw, Ann. Rev. Phytopathol., 1970, 8, 371.

201. C.G. Elliott and B.A. Knights, J. Sci. Fd. Agric., 1969, 20, 406.

202. E.H. Allen and J. Kuc, Phytopathol., 1968, 58, 776.

203. (a) P.A. Arneson and R.D. Durbin, Phytopathol., 1967, 57, 1358.
(b) Ident., Ibid., 1968, 58, 536.
(c) Ident., Plant Physiol., 1968, 43, 683.

204. E. Schlösser and D. Gottlieb, Arch. Mikrobiol., 1968, 61, 246.

205. J.W. Hendrix, R.D. Bennett and E. Heftmann, Microbios, 1970, 2, 11.

206. L.J. Morris, J. Lipid Res., 1966, 7, 717.

207. R.E. Gain, Mycologia, 1972, 64, 198.

208. C.G. Elliott, private communication.

209. P. Cuatrecasas, Adv. Enzymol., 1973, 36, 29.